A STABLE ISOTOPE APPROACH FOR THE EARLY DETECTION AND IDENTIFICATION OF N LOADING IN AQUATIC ECOSYSTEMS

Report to the
WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Global increases in urbanization and anthropogenic activity within watersheds and catchment areas have resulted in excessive nitrogen loads in aquatic ecosystems. South Africa is deeply dependent on natural resources for its economic health and as a consequence is particularly vulnerable to the degradation of its natural capital. Increased nitrogen loading can result in widespread aquatic ecosystem degradation including: harmful algal blooms, increased turbidity, hypoxia, loss of aquatic vegetation and habitat and fish kills, it is also one of the mechanisms driving aquatic weed invasions. Understanding the fate and processing of anthropogenic nutrients in natural systems is therefore critical for both preserving the well-being and biotic heritage for future generations as well as providing a tremendous opportunity to improve the management driven by science.

The objectives of this study were to evaluate the feasibility of mapping anthropogenic pollution through stable isotopes signatures of aquatic plants, to investigate the potential for identifying different pollution sources, concentrations and distributions in a freshwater environment and to determine the utility of these techniques in indentifying early eutrophication.

After field trials were complicated by the absence of chosen indicator species (*Eichhornia crassipes* & *Nasturtium officinale*) at numerous sites and the confounding effects of rooted individuals in comparison with free floating ones, it became clear that successful mapping of anthropogenic sources of nutrients in natural systems required a single aquatic plant species that could be easily transplanted from the lab to the field. *Spirodella* sp. was identified as being an appropriate alternative. This type of monitoring required a return to laboratory experiments to generate baseline data, but was clearly the way forward for pollution mapping through primary producers and the next step in understanding the mechanisms driving eutrophic systems.

Laboratory experiments involved determining isotopic tissue turnover times and quantifying isotopic relationships relative to increasing nutrient concentrations. Following these experiments this technique was field tested in local stream to confirm the ability of *Spirodella* sp. to reflect nutrient changes in a natural environment.

Results from these studies indicate that through the use of *Spirodella* sp. as an indicator species, nitrogen mapping, the identification of pollution hotspots and the monitoring of water quality can be done easily, in a fast and time-integrated fashion. *Spirodella* sp. clearly differentiated between different nutrient types within four days of exposure and established that not only do plants demonstrate concentration level isotope relationships, but transplanted individuals will reflect nutrient loading in a natural environment.

This technique of placing organisms into the field to monitor changes in nutrient status within a system has rarely been used in South Africa and has great potential. Transplantation to monitor differences in isotope signatures associated with changes in nutrient loading is still in its infancy and will require further fine-tuning. This technique needs to be field tested on a larger scale over a number of well defined nutrient gradients and further investigation into local/regional variation in nutrient sources needs to be completed. Additional laboratory studies to disentangle the effects of iron limitation on nutrient uptake with respect to isotope signatures may also provide insights into nutrient dynamics of eutrophic systems. The techniques investigated in this study show great potential for identifying pollution sources, concentrations and distributions within aquatic ecosystems. As these techniques develop, they may also afford water resource mangers the opportunity to address the causes of degrading ecosystems and aquatic weed invasions.

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1.0 Introduction

The excessive addition of nitrogen to watersheds is recognized as one of the main causes for the global deterioration of aquatic ecosystems (Rabalais 2002). Impacts of surplus nitrogen in waterways include hypoxia, fish-kills, toxic algal blooms, the disruption of ecosystem functioning, loss of diversity and the establishment of invasive species (e.g. Vitousek et al. 1997, Rabalais 2002, Green & Galatowitsch 2002). Furthermore, high nitrate concentrations are also recognized as posing a direct health risk to human consumers (WHO, US EPA). With many of South Africa's waterways highly eutrophic (Walmsley 2000), nitrogen pollution emanating from commercial and domestic sewage as well as agricultural run-off poses a major threat.

Current assessment strategies to evaluate ecosystem health include the monitoring of taxonomic shifts in the abundance of the aquatic biota. While these biological indexes have proven useful both globally and locally (through the River Health Programme), bio-monitoring by its very nature, typically identifies eutrophication problems only once ecosystem-level impacts have already occurred. Furthermore, where ecosystem health has been disrupted, it is often not possible to link the changes in the biota to identifiable causes (especially where pollutants originate from non point-sources). Any methods that would allow for the detection of incipient eutrophication as well as being able to trace and identify nutrient sources would therefore greatly add to our ability to effectively manage our aquatic resources.

In recent years, an increasing number of studies have shown that stable isotope signatures of nitrogen ($\delta^{15}N$) in aquatic biota reflect the 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). This is due primarily to isotope fractionation resulting in the progressive concentration of the lighter ^{14}N isotope in the biota during periods when nitrogen is not limiting (Kendall & Doctor 2003). Furthermore, much progress has been made in isotopically distinguishing nitrogen sources such as sewage, manure and synthetic fertilizers, as the isotopic signatures of these substances reflect the pathways by which they were created (e.g. Curt et al. 2004, Kendall 1998, Reynolds-Vargas 2006, Van der Zanden et al. 2005).

In South Africa, stable isotopes have successfully been used in aquatic environments to investigate groundwater recharge and hydrology, to monitor aquatic food webs and to define riparian zones. To our knowledge, however, no concerted effort has as yet been made to test the utility of stable isotopes as indicators of early eutrophication or as a tool to identify and trace pollutants in aquatic ecosystems. The proposed research aims to investigate whether $\delta^{15}N$ signatures in aquatic primary producers can be used in a local setting to determine the nutrient status of ecosystems and whether local N-sources (e.g. synthetic fertilizers, manure and sewage) can be isotopically differentiated and their spatial pathways mapped through the waterways.

1.1 Impacts: Environment

The proposed research is a first attempt at testing and developing a stable isotope monitoring tool that has the potential of greatly enhancing our ability to manage our aquatic ecosystems. In the

longer term, the methodology should allow us to identify the eutrophication status of freshwaters, to provide an early warning system for deteriorating watershed quality, to determine the origin of nitrogen sources, to investigate pollutant pathways through our environment and to identify sites of management interest (e.g. identifying polluters or important sites of denitrification).

1.2 Aims of the Study

1) To test the utility of using $\delta^{15}N$ in aquatic primary producers to monitor the nutrient status of aquatic systems.
2) To test whether major nitrogen sources can be isotopically distinguished (using $\delta^{15}N$ and $\delta^{13}C$).
3) To investigate the feasibility of tracing mixed nutrients back to their various sources of origin.
4) To quantitatively determine the relationship between nutrient loading and the isotopic composition of indicator species.

2.0 Methodology

2.1 Background to the stable isotope methodology

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 mass difference 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 isotope signature (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. This results in enriched nitrogen isotope signatures of plant tissues (+2 permil to +4 permil) in areas of high nitrogen loading. Elevated nitrogen isotope signatures in plants (but also consumers) are therefore a good indicator of high nitrogen loading (eutrophication). Unlike direct nutrient assays, organismal nitrogen isotope signatures provide a time-integrated signal which depends on the tissue turn-over time (days, weeks or months) and the assimilation rate of the organism under investigation. Nitrogen isotope signatures can therefore provide a more integrated assessment of the nitrogen-loading of a site than the spot-analyses of nutrients which may be affected by temporal variability in the nutrient discharge regime and/or the flow regime of the river. In addition to acting as an indicator of nitrogen loading, stable isotope signatures can be a useful tool for tracing nitrogen sources in watersheds (sometimes this is done in combination with other isotopes). Plants assimilating nitrogen from synthetic fertilizers have nitrogen isotopes signatures that reflect the atmospheric N_2 source of the fertilizer (-2 permil to +2 permil). In contrast, organic nitrogen sources such as manures and sewage have a very different isotopic composition (typically +10 permil to +25 permil) that reflects the pathways by which they were created. Accordingly, by mapping the isotope signatures of a plant species in a particular watershed, it is often possible to determine both the spatial source and the composition of nitrogen sources. This technique, sometimes referred to as sewage plume mapping, 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, watersheds, estuaries and coastal environments. Isotope analyses of $\delta^{15}N$ and $\delta^{13}C$ are typically performed in unison and can be performed at several Isotope Ratio Mass Spectrometer laboratories in South Africa. Field collection of tissues is a simple process which requires little to no expertise. After collection, samples require to be stored frozen or dried before dispatching them to the lab for analysis.

2.2 Background to the choice of sites and indicator organisms

As the aims of this project include the development of a rapid monitoring tool for the nutrient status of aquatic ecosystems, it was decided that primary producers should be used as indicator organisms. In the past, invertebrate indicator species have also been used successfully, but due to their higher trophic level and slower tissue turn-over rates, their isotopic responses to nutrient fluxes tend to be less rapid than for primary producers. Originally the free-floating and invasive water hyacinth (*Eichhornia crassipes*) and a submerged waterweed (*Potamogeton* sp.) were chosen as test organisms. Water hyacinth for example represents a major problem species in South Africa, the proliferation and/or management success of which has been linked to the nutrient status of the system it inhabits (Hill and Olckers 2000, Coetzee et al. 2006). As it is free floating, it should serve as an ideal indicator of the N-loading of the water that surrounds it. *Potamogeton pectinatus* was chosen as it represents an indigenous taxa that has the potential of acting as an indicator species throughout the region. Unfortunately *Potamogeton* spp, although prevalent in dams and impoundments, does not grow well in faster moving waters (Henderson & Cilliers 2002) and the second indicator species was subsequently changed to the edible watercress (*Nasturtium officinale*), a declared invader which grows well in variable flow regimes. Ultimately however, the absence of both water hyacinth and/or watercress in many sampling areas, and the logistical difficulties in finding plants spanning variable nutrient regimes, resulted in a change to a third indicator species, the cosmopolitan free-floating *Spirodella* spp.

While there is some evidence to link water nutrient status with the invasiveness of floating aquatic macrophytes (Hill and Olckers 2000), there remains much controversy on the effect of water nutrients on submerged species (Thomaz et al. 2007). While it is not the purpose of this study to specifically work on invasive species, the choice of the presented indicator species will (in addition to testing the isotope monitoring tool) facilitate a better understanding of plant/nutrient interactions required for the effective control of invasive plants. The initial investigation of the utility of the isotope method to monitor and identify pollution sources will be carried out on two different Eastern Cape impoundments and their tributaries and outflows, the New Year's River and dam near Alicedale and the Kubusi River and Wiggleswade Dame near Stutterheim. Both systems formed part of the WRC project "Integrated management plan for the biological control of water hyacinth" (TT 454/10) and monthly background information on nutrients exists for at least two years. Furthermore, the River Health Programme is shortly due to start monitoring the Bushmans River (part of the New Years Dam system). Investigation of the selected sites will add to our understanding of environmental water quality issues and their potential impacts in the proposed system. New Year's Dam (close to Alicedale) is a relatively healthy oligotrophic system with no identified direct point-source nutrient inputs. Water hyacinth for example, has been successfully controlled on this dam through the introduction of the biological control agent, *Neochetina eichhorniae*. The success here and not in a number of other systems has been attributed to the low nutrient status of the dam. Downstream of the dam, however, N point-

sources exist in the form of sewage inputs from the Alicedale settlement and the existence of a golf-course. This system therefore provides us with an ideal scenario to investigate the effect of nutrient concentrations (i.e. dilution away from the sources) on the isotopic composition of primary producers. The Kubusi River and Wriggleswade Dam (near Stutterheim) and its tributaries are heavily impacted and represent a eutrophic system with large- scale diffuse agricultural inputs as well as with a sewage point-source at Stutterheim. In this system, the mixing of several ill defined nitrogen sources, provides us with the opportunity to study the utility of stable isotopes in identifying individual nitrogen sources in a mixture and mapping their pathways and assimilation by the biota.

2.3 Sampling and analysis:

Aim 1:

Between the two systems, four N point sources have been identified which can be used to investigate the isotopic composition of primary producers under different N loading regimes (distances upstream and downstream from the sources). At each of these point sources, six samples of primary producers will be collected (2x upstream, 1x at source, 3x downstream) together with water samples for nutrient analysis. As plant tissues have in the past shown some variability in isotopic composition, tissue samples will be restricted to the growing tips and will be collected in triplicate. As both the flow regime of the river and the input of nutrients may vary over time, it is suggested that sampling should occur twice a year, once during the wet and once during the dry season. The resulting data should allow us to test whether the isotopic composition of primary producers is a good indicator of the nutrient status of the environment that they inhabit. Furthermore, we will also gain information about intra and inter-species variation in isotope assimilation and assess the potential importance of temporal variability.

Aim 2**:**

During the initial investigation of nutrient gradients, point sources will have been identified and the isotopic composition of the primary producers closest to the sources will have reflected those of the effluent. Where possible direct effluent samples will be collected (particulate and dissolved) to test whether different source types can be isotopically distinguished (e.g. sewage, manure, synthetic fertilizers). Where possible, additional samples of the same types will be collected from other point sources in the region to determine whether source types are statistically and isotopically distinct.

Aim 3**:**

Originally this aimed to use two sites within the identified systems where different nutrient sources mix (fertilizer & sewage; sewage and agricultural run-off). Upstream and downstream of these sources and mixing sites, it would be attempted to model the pathways, dilution and assimilation of the nutrient types in the indicator species

by using an isotope mixing model (IsoSource, US EPA). For this purpose, 10 sampling sites would have been established along a longitudinal transect (three replicates each) along each mixing zone and samples analysed for both carbon and nitrogen isotopes. It was also suggested that the sampling design be conducted once during the wet and once during the dry seasons, as the flow regime of the rivers is likely to affect nutrient mixing. Unfortunately, after exhaustive field surveys it became apparent that neither watercress nor water hyacinth were present in enough sites across any nutrient gradients, or indeed both upstream and downstream of pollution sources to allow any type of modelling. This resulted in (as outlined in deliverable 2) switching the indicator species to the free floating *Spirodella* spp, which lead to investigating the potential of using *Spirodella* sp. in transplantation experiments that could address nutrient mixing in the systems where water hyacinth and watercress could not. Here this study aims to pilot an investigation into transplanting plants of known isotope signatures into the natural environment and determining to what extent stable isotopes in indicator species reflect the nutrient status and composition of their environment.

Aim 4**:**

The above field sampling will have established whether and to what extent stable isotopes in indicator species reflect the nutrient status and composition of their environment. The quantitative relationship between isotope signatures and nutrient concentrations is however more difficult to establish in the field. As indicated earlier, stable isotope signatures provide a time-integrated assessment of nutrient status whereas nutrient assays in the field are spot measurements. The two cannot therefore be easily related as the spot measurements are likely to be far more variable that the isotope signatures. This problem may be resolved by laboratory manipulations. It is suggested that the two indicator species be grown in the laboratory under seven different but stable nutrient regimes. This should allow for an assessment of the quantitative relationship between the two parameters. Furthermore, by sub-sampling the indicator species prior to and at different times during the experiment, it should be possible to quantify how fast their isotopic composition reacts to changes in their environment.

All samples of solid and particulate matter will be analysed at the IsoEnvironmental Isotope Facility at Rhodes University. Where liquid samples require analysis, samples will be sent to the Ithemba National Isotope Laboratory.

3.0 Literature Review: Stable Isotopes in Aquatic Ecosystem Monitoring and Nutrient Source Determination: A Review

3.1 Introduction

Global increases in urbanization and anthropogenic activity within watersheds and catchment areas have led to excessive addition of nitrogen to aquatic ecosystems, resulting in the

deterioration of many streams, rivers, lakes and coastal environments (Howarth 1998; Rabalais 2002; Anderson & Cabana 2005). For example, in the Baltic catchments of the North Atlantic Ocean (Galloway *et al*. 1996) and the watersheds of the Mississippi River (Mitsch *et al*. 2001), inputs of anthropogenic nitrogen now greatly exceed, and in some cases double those from natural inputs (i.e. atmospheric deposition and nitrogen fixation). Increased nitrogen loads can have wide ranging effects in aquatic environments which may result in widespread ecosystem degradation, including, but not limited to: food web shifts and disruption of ecosystem functioning (Spies et al. 1989; McClelland & Valiela 1998b; Deegan et al. 2002; De Bruyn et al. 2003), loss of biodiversity (Roberts et al. 1996; Vitousek *et al*. 1997; Rabalais 2002; Anderson & Cabana 2005), harmful algal blooms (HABs; Hodgkiss & Ho 1997), fish kills (Tveite 1984), increased turbidity and hypoxia (Zimmerman & Canuel 2000), loss of submerged aquatic vegetation and habitat (Smith 1998; Carpenter *et al*. 1998; Tewfik et al. 2007) as well as the colonization and dominance of invasive species (Green & Galatowitsch 2002). Furthermore, high loads of nitrate are recognized as a significant health risk to human consumers (WHO, US EPA). Understanding the fate and processing of anthropogenic nutrients in natural systems is therefore essential to monitoring water quality and aquatic ecosystem health.

In the past, aquatic ecosystem health has been monitored through taxonomic changes in the abundance of aquatic biota [e.g. Index of Biotic Integrity (IBI)]. While these biological indices have proven useful both globally and locally, bio-monitoring by its very nature, typically identifies eutrophication problems only after the ecosystem-level impacts have taken place. Furthermore, where ecosystem health has been disrupted, it is often difficult to identify the mechanisms behind changes in IBI, especially where pollutants originate from non point-sources. As such, the search for new indices continues, specifically those that would allow for the detection of incipient eutrophication as well as being able to trace and identify nutrient sources in order to improve our ability to effectively manage our aquatic resources. With many of South Africa's waterways already having eutrophication problems, nitrogen pollution emanating from commercial and domestic sewage as well as agricultural run-off pose major threats.

A large component of eutrophication in aquatic systems can be attributed to anthropogenic inputs of sewage nitrogen (Lee & Olsen 1985; Nixon et al. 1986; Constanzo et al. 2001) and the management and monitoring of affected environments has become a major environmental problem (Nixon 1995). Mapping the distribution of sewage effluent in aquatic systems has been undertaken using a variety of different techniques, from organic matter composition (e.g. Reina et al. 2006), radioisotope tracers (e.g. Lagerwerff 1976; Dureth et al. 1986), dye fluorescence (e.g. Stedmon et al. 2003; Hudson et al. 2007), dissolved nutrients (Waldron et al. 2001), tissue N content (Fong et. al. 1998) salinity (Middelburg & Nieuwenhuize 2001), bacteria (Wakelin et al. 2008), and $\delta^{15}N$ signatures of water and sediment (Lindau et al. 1989; Sweeney et al. 1980; Smith-Evans and Dawes 1996). However, in well mixed systems the environmental signal of these tracers are rapidly lost, providing, at best, only an instantaneous view of sewage dispersal and short pulses of sewage pollution (specifically in areas that may not normally receive inputs) are likely to be missed, although these events may be ecologically significant (Gartner et al. 2002). Additionally, while these techniques are useful for determining the physical extent of sewage presence, they provide little insight into the biological uptake and influence of sewage nutrients within an ecosystem (Constanzo et al. 2001). Furthermore, these parameters are often confounded by spatial and temporal variability.

3.2 Stable Isotope Applications

A number of studies using stable isotope analysis (SIA) have definitively shown the assimilation of sewage particles into aquatic food webs, ranging from marine embayments (Rau et al. 1981; Rogers 1999; Tucker et al. 1999; Waldron et al. 2001) and coastal environments (Sweeney et al. 1989; Spies et al. 1989) to incorporation into deep-sea food webs (Van Dover 1992). Anthropogenic N derived from septic tanks has also been traced using elevated $\delta^{15}N$ signals into stream biota through groundwater contamination in an urban watershed (Steffy & Kilham 2004) and into phytoplankton and macroalgae in a coastal bay (McClelland & Valiela 1997). More recently, research has shown that stable isotope signatures of nitrogen ($\delta^{15}N$) in aquatic biota reflect the 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). Primary consumers such as clams (Fry 1999), oysters (Piola et al. 2006) or mussels (Cabana & Rasmussen 1996; Lake et al. 2001; McKinney et al. 2001; Fry & Allen 2003; Moore & Suthers 2005) for example, have been used as bioindicators of N-loading in lake and river/estuary watershed linkages, suggesting that these animals reflect long-term effects of anthropogenic catchment disturbance and nutrient enrichment. Similarly, stable nitrogen ratios of a number of fish species were employed by Cabana & Rasmussen (1996), Wainright et al. (1996), Harrington et al. (1998), Lake et al. (2001), Gaston & Suthers (2004) and Schlacher et al. (2005) as a description of eutrophication that could be correlated to urban development wastewater pollution in both freshwater and estuarine systems. Investigations into the role of sewage nitrogen in littoral food webs has also been attempted using macroinvertebrates (De Bruyn & Rasmussen 2002; De Bruyn et al. 2003) and isotopic studies on an insectivorous bird species, *Tachycineta bicolor* suggested that anthropogenic N tracing may be extended to riparian species feeding on aquatic prey (Wayland & Hobson 2001). Stable nitrogen isotopes from higher vertebrate species such as feathers from flightless mallard ducklings (*Anas platyrhynchos*) have also been shown to reflect long-term nitrogen additions to surface waters in agricultural areas, as well as elucidating the origin of non-point source N inputs (Hebert & Wassenaar 2001). There are however, a number of pitfalls associated with such measurements of anthropogenic pollution. Firstly, reflections of long-term exposure to anthropogenic nitrogen additions face similar drawbacks to those associated with IBI, namely that animals that grow slowly relative to the pollution inputs are likely to show isotopic change only after ecosystem level alterations have taken place. Secondly, isotopic measurements in these animals represent an average signal over time and consequently will not adequately reflect temporal variation in anthropogenic influence. Lastly, many of these species (with the exception of bivalves) are highly mobile, whether it is from system to system or within a system, which complicates an assessment of the spatial influence of pollution as well as distinguishing point and non-point sources of anthropogenic inputs.

It is clear however, that higher (increased $\delta^{15}N$) nitrogen isotope signatures (relative to baseline values) can be strongly correlated with human activities in watersheds, especially conversions from natural to agricultural lands and those associated with increasing urbanization (Cabana & Rasmussen 1996; Harrington et al 1998; Hebert & Wassenaar 2001; Udy & Bunn 2001). N-nutrients are flushed downstream from developing watersheds most often in the form of nitrate (Cole et al. 1993; McClelland & Valiela 1998a), and the $\delta^{15}N$ ratios of this nitrate frequently increase with human population density (Fig 1, reproduced from Cabana & Rasmussen 1996).

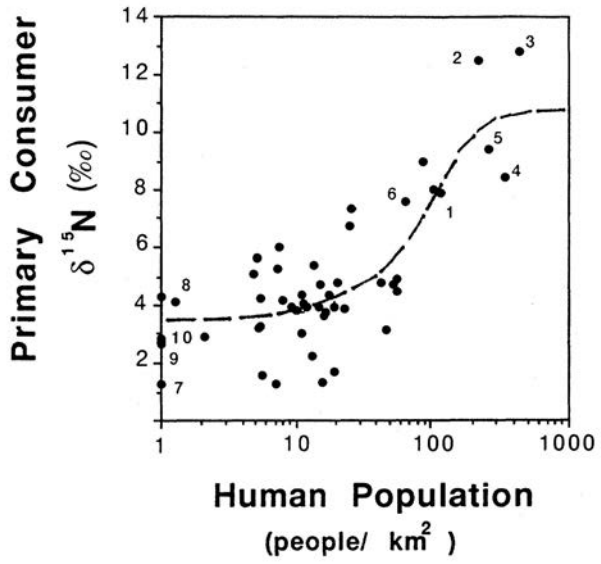


Figure 3. 1: Variation in the δ^{15}N signature of primary consumers reflects the human population density of the watershed, likely a reflection of the high ^{15}N content of domestic sewage. Reproduced from Cabana & Rasmussen 1996.

3.3 Source Determination

Macroalgae depend primarily on dissolved inorganic nitrogen (DIN) from the water column to meet their nitrogen requirements (Wallentius 1984). As such, the fractionation accompanying nitrogen uptake as well as the source of the DIN will determine an alga's isotopic signature. The first factor, the fractionation accompanying N uptake, provides an early warning component to isotope studies in aquatic systems. This is due primarily to isotope fractionation resulting in the progressive concentration of the lighter ^{14}N isotope in the biota during periods when nitrogen is not limiting (Kendall & Doctor 2003). When nitrogen is limiting, all available nitrogen is assimilated and the resulting plant tissues exhibit an isotope signature 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. This results in enriched nitrogen isotope signatures of plant tissues in areas of high nitrogen loading (Kendall 1998; Kendall & Doctor 2003). Elevated nitrogen isotope signatures in plants (but also consumers) are therefore a good indicator of high nitrogen loading (eutrophication). Unlike direct nutrient assays, organismal nitrogen isotope signatures provide a time-integrated signal which depends on the tissue turn-over time (days, weeks or months) and the assimilation rate of the organism under investigation. Nitrogen isotope signatures can therefore provide a more integrated assessment of the nitrogen-loading of a site than the spot-analyses of nutrients which may be affected by temporal variability in the nutrient discharge regime and/or the flow regime of the river. Plant indicators may then act as sentinels enabling early detection of high N loading in the environment. These biological indicators can therefore detect whether a community is under threat prior to an observed decline in ecological health and enables the early initiation of management practices.

In addition to acting as an indicator of nitrogen loading, stable isotope signatures can be a useful tool for tracing nitrogen sources in watersheds. The second factor, the source of the DIN, can also be responsible for elevated values of $\delta^{15}N$ in marine plants. In cases where nitrogen is limiting for example, algae will often assimilate nitrogen additions from alternative sources and recently, elevated $\delta^{15}N$ signatures have been identified in marine plants exposed to septic contaminated groundwater (McClelland et al. 1997), seabird guano (Wainright et al. 1998), shrimp farm (Costanzo et al. 2004) and sewage effluent (Dhargarlker 1986; Hobbie et al. 1990; Handley & Raven 1992; Lyngby & Mortensen 1994; Cabana & Rasmussen 1996; Grice et al. 1996; Hansson et al. 1997; Udy & Dennison 1997; McClelland & Valiela 1998a). In the cases of Monterio et al. (1997) and Anderson et al. (1999) the $\delta^{15}N$ levels in macroalgae were been used to indicate anthropogenic N contributions related to macroalgal blooms in the vicinity of a fish-processing waste outlet. The tracing of anthropogenic N is often accomplished in combination with other isotopes such as carbon (e.g. Waldron et al. 2001; Wayland & Hobson 2001; Rogers 2003; Piola et al 2006) and in a few cases, sulphur (Sweeney & Kaplan 1980; Sweeney et al. 1980; McCarthy et al. 1997; Wayland & Hobson 2001; Fry & Allen 2003). Accordingly, by mapping the isotope signatures of a plant species in a particular watershed, it is often possible to determine both the spatial influence and the composition of nitrogen sources. This technique, sometimes referred to as sewage plume mapping, 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, watersheds, estuaries and coastal environments.

Furthermore, much progress has been made in isotopically distinguishing nitrogen sources such as synthetic fertilizers and sewage from natural inputs as the isotopic signatures of these substances reflect the pathways by which they were created (e.g. Curt et al. 2004, Kendall 1998, Reynolds-Vargas 2006, Van der Zanden et al. 2005). For example, rivers with low anthropogenic N loads have a DIN signature of < 8‰, reflecting nitrate and ammonium sources from atmospheric deposition and/or nitrate from nitrification in pristine soils (McClelland & Valiela 1998a), while the main method of nitrate and ammonium fertilizer production is through the industrial fixation of atmospheric nitrogen, resulting in $\delta^{15}N$ signatures closer to zero (0 ± 3‰; Table 1). Organic nitrogen sources such as treated sewage are different again, with isotopic compositions (typically +10 to +25 ‰; Table 1) that reflect the natural microbial fractionation processes in tertiary sewage treatment plants, which are strongly discriminatory, selectively using ^{14}N and producing ^{15}N-enriched wastewater (Savage et al. 2004; Constanzo et al. 2005). When nitrogen is excreted in animal or sewage waste, it is typically in the form of urea, which when hydrolyzed produces a temporary rise in pH. These more basic conditions favour the conversion to ammonia, which is subsequently volatized to the atmosphere. Fractionation during this process results in isotopically depleted (in ^{15}N) ammonia (which is lost from the system) and the correspondingly enriched ammonium is then converted into ^{15}N-enriched nitrate. This set of processes results in sewage nitrogen being readily distinguishable from other nitrogen sources (Heaton 1986; Constanzo et al. 2001).

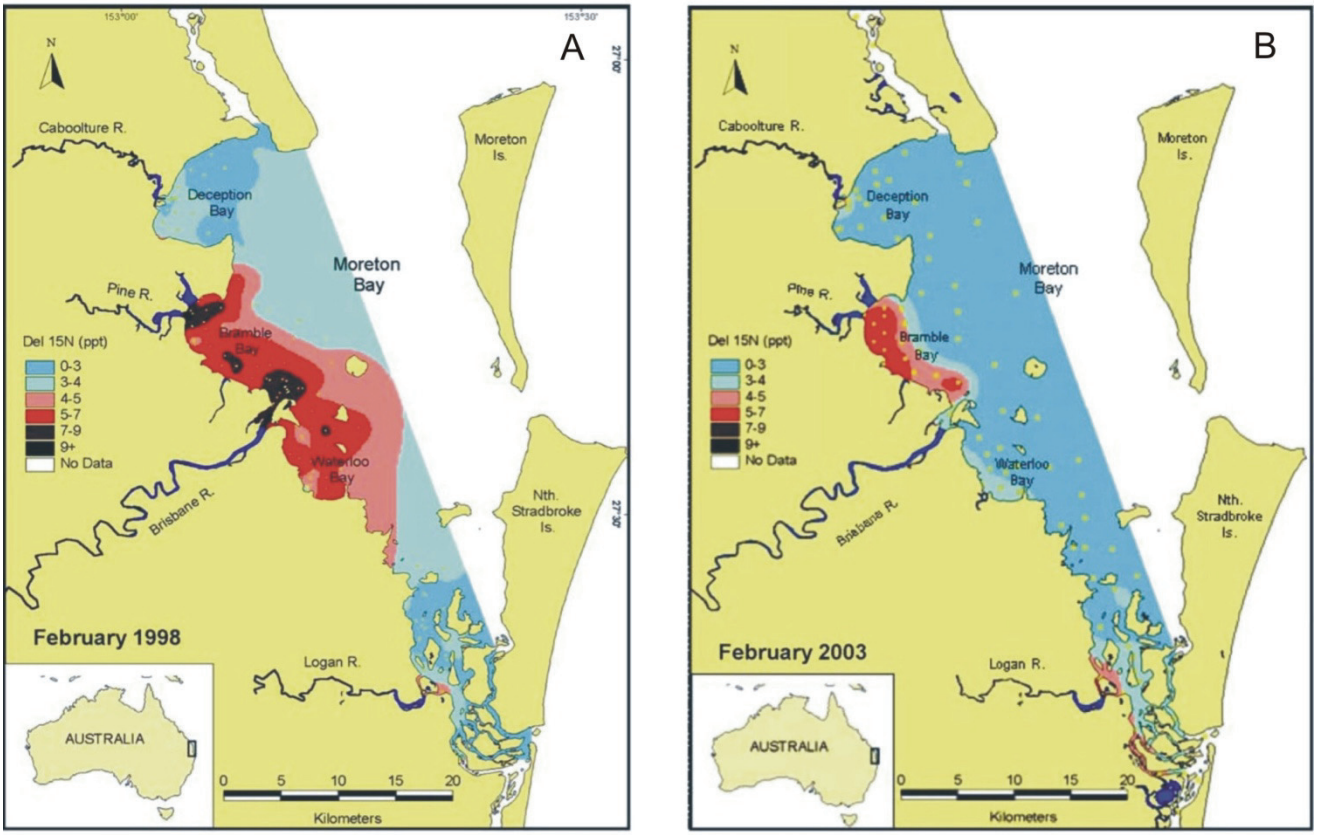
Table 3. 1: Reported $\delta^{15}N$ signatures (‰) for different sources of dissolved inorganic nitrogen (DIN).

Source of DIN	**$\delta^{15}N$ (‰)**	**Reference**
Synthetic fertilizer	-1.6 1.48 -3 to 3 0	Widory et al. 2004 Curt et al. 2003 Kendall 1998; Heaton 1986; Kreitler & Browning 1983; Kreitler et al. 1978; Kohl et al. 1971
Groundwater N (atmospheric deposition)	<5 <8 2-8	Mayer et al. 2002 McClelland & Valiela 1998 Kreitler & Browning 1983; Kreitler et al. 1978
Nitrate (NO_3^-) derived from raw sewage	4.3-8.4	Widory et al. 2004
Wastewater DIN (NH_4^- + NO_3^- e.g. treated sewage)	16 ± 2.3 13.5-25.3 11.52 10-22	Piola et al. 2006 Curt et al. 2003 Gartner et al. 2002 Jordan et al. 1997; Macko & Ostrom 1994; Aravena et al. 1993; Owens 1987; Heaton 1986; Kreitler & Browning 1983; Kreitler et al. 1978
Nitrate (NO_3^-) derived from animal manure	5-35 15.98 10-25	Widory et al. 2004 Curt et al. 2003 Kendall 1998

3.4 Spatial Mapping of Anthropogenic Nitrogen

Recently, the development of an innovative technique using $\delta^{15}N$ signatures in marine plants to provide temporally integrated information on the biologically available (and hence ecologically important) component of anthropogenic nitrogen has come to light. Pioneered by Costanzo et al. (2001) and refined by Constanzo et al. (2004; 2005), this technique used changes in macroalgae $\delta^{15}N$ to map the fate of sewage derived N within Moreton Bay, QLD, Australia. Moreton Bay is a sub-tropical shallow coastal embayment on the east coast of Australia with a drainage catchment (2.1×10^4 km^2) containing an urban center with a population of approx. 1.5 million people. Development is concentrated on the western side of the bay, and the majority of treated sewage effluent is discharged in four river estuaries also on the west side (~2000 tonnes nitrogen/year in the Brisbane River estuary alone; see Fig 2C), while the east side of the embayment receives

low-nutrient oceanic water and few anthropogenic nutrients (Udy & Dennison 1997). Because using $\delta^{15}N$ signatures of naturally occurring plants is limited by their distribution, Costanzo et al. (2004; 2005) determined the nitrogen signatures of a fast growing, red macroalgae (*Catenella nipae*) on the bay's east side and then transplanted live plants from the east to the west side of Moreton Bay. *C*. *nipae* were incubated (for four days) at approx. 100 sites, deployed in a radiating grid pattern adjacent to the four major river mouths entering the embayment. Costanzo et al. (2005) employed this mapping technique in two different seasons (Sept 1997, Feb 1998), to quantify seasonal variations in pollution inputs and again over a five year period (1998-2003). Their transplantation technique provided exceptional maps demonstrating the spatial extent of sewage inputs in Moreton Bay and the reduction in algal $\delta^{15}N$ values correspond to drastic cutbacks in sewage loading by three of the four sewage treatment plants (in response to large government investments aimed at effluent N removal) adjacent to Bramble Bay (see Fig 2A-B, reproduced from Constanzo et al. 2005), demonstrating a reduction in bioavailable sewage nitrogen.



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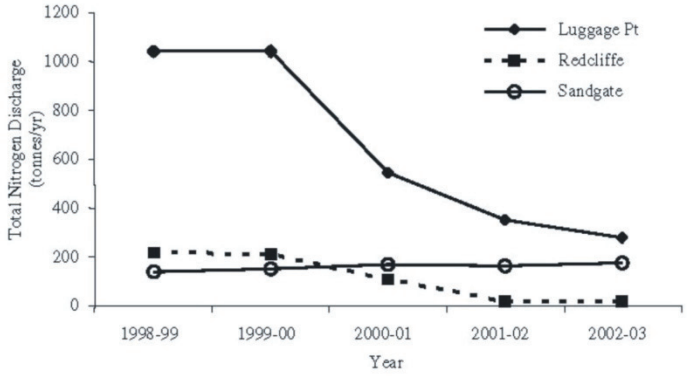

Fig. 2. Total annual nitrogen discharge (tonnes/year) from the three sewage treatment plants (Luggage Point, Sandgate, Redcliffe) adjacent to Bramble Bay from 1998 to 2003.

Figure 3. 2 Spatial and temporal distribution of deployed macroalgal $\delta^{15}N$ values in February (a) 1998; and (d) 2003. Macroalgae (*Catenella nipae*, Rhodophyte) was deployed at ~100 sites (yellow solid circles) in Moreton Bay, Australia. Reproduced from Costanzo et al. 2005.

Unsure of the reliability of this technique, Deutsch & Voss (2004) set out to determine if algal tissue directly reflects the $\delta^{15}N$ signature of river nitrate by collecting macroalgae from an unpolluted estuarine site and incubating them in a nitrogen rich estuary. Their results showed a robust relationship between nitrogen signatures of red and brown algae the $\delta^{15}N$ values of river nitrate (Fig 3; reproduced from Deutsch & Voss 2004), confirming that marine plants are reliable indicators of surrounding waters and can infer a degree of anthropogenic nitrogen in estuaries. Additionally, Cole et al. (2004) compared plant $\delta^{15}N$ values to the modeled wastewater nitrogen load for a number of estuaries. Her results show that a relationship exists between the isotopic signal of groundwater and producers, and the wastewater nitrogen load value derived from the model (see Fig 4, reproduced from Cole et al. 2004). This relationship can be used to predict what percentage of nitrogen (i.e. all the nitrogen coming into an estuary) is coming from wastewater via groundwater.

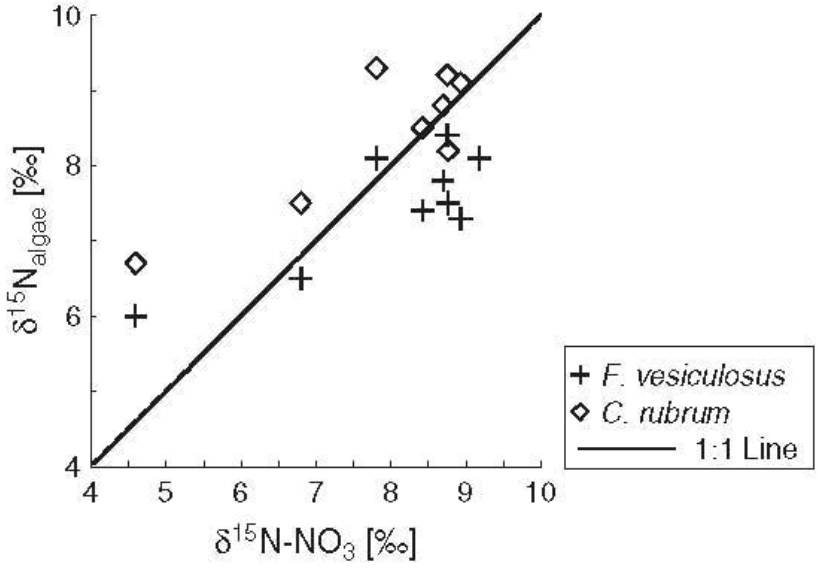

Figure 3. 3 $\delta^{15}N\text{-}NO_3^-$ values vs. $\delta^{15}N$ values of macroalgae. The 1:1 line indicates the theoretical $\delta^{15}N$ value of the macroalgae, if the $\delta^{15}N$ value of the nitrate was mirrored exactly. Reproduced from Deutsch & Voss (2004).

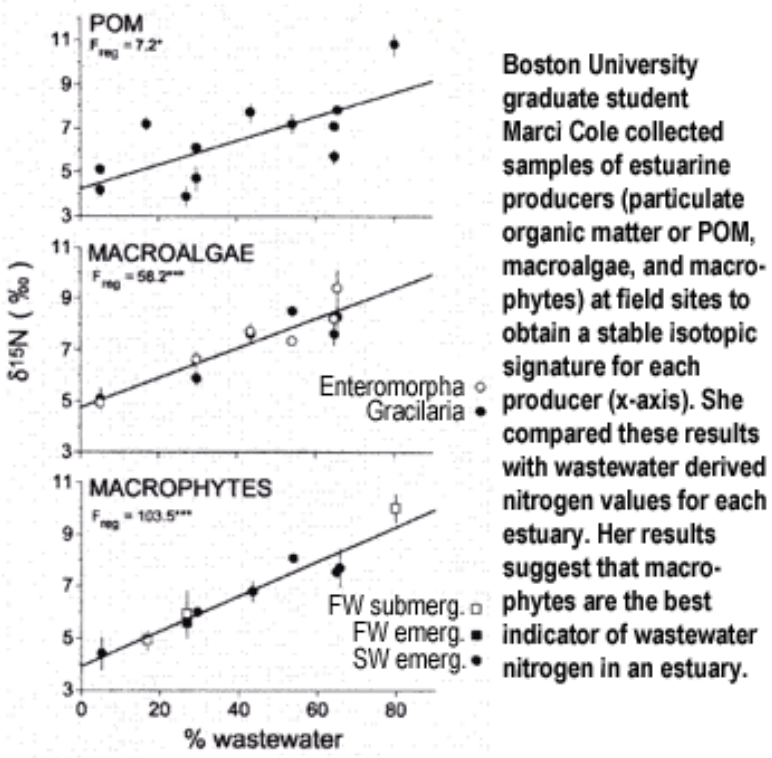

Figure 3. 4: Reproduced from Cole et al. 2004

A similar approach to sewage plume mapping, recently employed by a number of studies, involves using the $\delta^{15}N$ ratios of naturally occurring marine plants taken at various distances from the anticipated source of anthropogenic N. This technique has been applied on a number of occasions, most notably to investigate the spatial influence of sewage outfalls through $\delta^{15}N$ measurements of *Fucus vesiculosus* in Himmerfjärden Bay, Sweden (Savage & Elmgren 2004; Savage 2005). Spatial and temporal variation of isotopic ratios in naturally occurring plants not only demonstrated decreasing $\delta^{15}N$ with distance from the sewage outfalls, but also showed a marked reduction in response to reduce N loading, implying that the proportion of sewage-derived N assimilated by the algae had declined and that the spatial influence of sewage has been reduced since the initiation of tertiary N treatment (Fig 5, reproduced from Savage 2005). A more in-depth approach by Gartner et al. (2002) used different functional forms of macroalgae to reveal temporal and spatial patterns in sewage dispersal in highly mixed, nitrogen limited waters. They demonstrated a relationship between $\delta^{15}N$ ratios and functional form, suggesting that $\delta^{15}N$ signatures of macroalgae with different nutrient uptake characteristics can provide integrated pictures of sewage influence over different timescales.

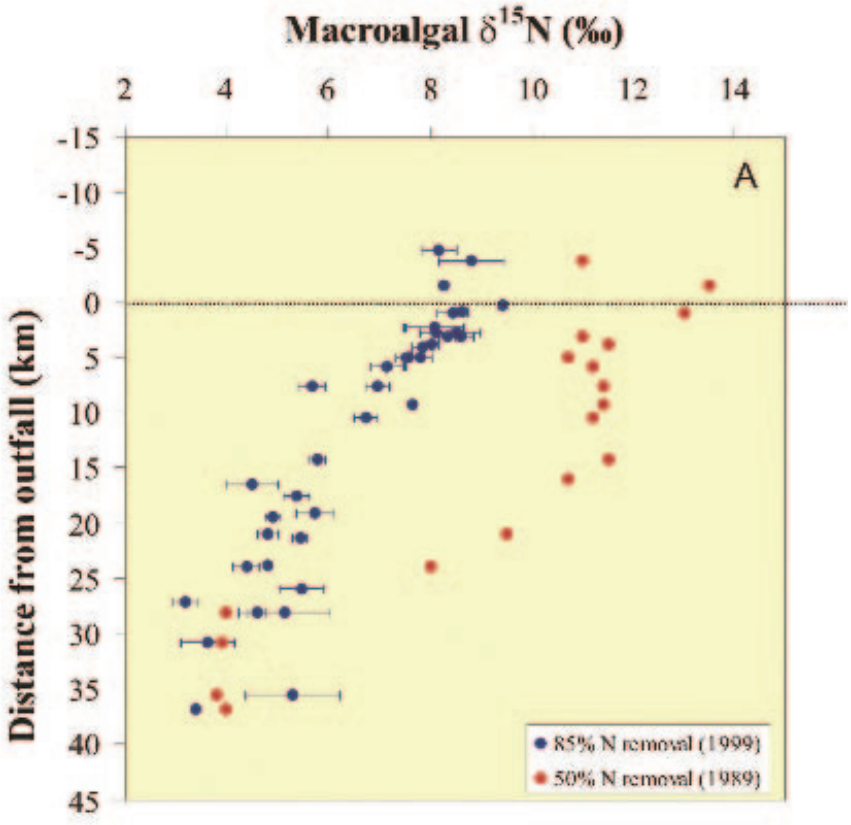

Figure 3. 5: Macroalgal $\delta^{15}N$ values in 1989 (with 50% N removal in the sewage treatment plant) and in 1999 (following 85% N removal in the sewage treatment plant) in relation to distance from the outfall (km). Reproduced from Savage 2005.

Other applications of anthropogenic nitrogen mapping through aquatic plant $\delta^{15}N$ ratios include the assessment of: ecological impacts of shrimp and sewage effluent (Jones et al. 2001); the impact of point source pollution on nitrogen isotope signatures of vegetation in SE Brazil (Stewart et al. 2002) and N loading on wide geographic scales, from North to South America (Cole et al. 2004); in Tomales Bay, California (Fourqurean et al. 1997); in the Baltic Sea (Deutsch & Voss 2004); in Portuguese estuaries (Castro et al. 2007); in Narragansett Bay, Rhode Island (Pruell et al. 2006); along the barrier island in Brevard and Indian River County, FL (Barile 2004) and within coral reef ecosystems (Lapointe 1997; Umwezawa et al. 2002; Yamamuro et al. 2003; Lin et al. 2007). According to Carpenter et al. (1998b) a system experiencing increased N loading can recover by decreasing input rates of N to aquatic ecosystems, but the rates of recovery are highly variable among water bodies, although often, the eutrophic state is persistent, and recovery is slow. However, Rogers (2003) was able to quantify recovery of marine biota after the closure of a sewage outfall at Moa Point, New Zealand. Furthermore, these techniques are not only limited to aquatic systems, but can also be as a means of identifying buffer zones where denitrification is actively processing allochthonous nitrate in riparian ecosystems and their surrounding catchments (Clement et al. 2003).

The majority of sewage plume mapping through $\delta^{15}N$ ratios of aquatic plants has been done in marine and estuarine systems, where anthropogenic N inputs have been identified as serious culprits in aquatic eutrophication. However, to date, little comparable work has been done in freshwater systems (De Bruyn & Rasmussen 2002). A very recent study by Benson et al. (2008) has attempted to address this issue, and set out to assess the anthropogenically derived N inputs to the Upper Saranac Lake, NY in the context of two additional water bodies that vary along a gradient of watershed population density. Their results suggest that not only do marine plants provide consistent eutrophication indicators, but freshwater angiosperms (i.e. *Vallisneria Americana*) reliably show variation in septic inputs to the Upper Saranac Lake, with some areas of the lake receiving more input than others. Additionally their results also confirmed that increased watershed population density is correlated with elevated $\delta^{15}N$ angiosperm signatures, as previously described for marine and estuarine environments (Cole et al. 1993; Cabana & Rasmussen 1996; Harrington et al 1998; McClelland & Valiela 1998a Hebert & Wassenaar 2001; Udy & Bunn 2001).

3.5 Conclusions

The increasing use of the world's freshwater resources, coupled with the acknowledged environmental deterioration and exhaustive use of limited resources calls for changes in present and future urban water and wastewater systems (Morrison et al. 2000). An even larger problem is facing developing countries (like South Africa) with moderate or heavy stress on their freshwater resources. To date, no aquatic bio-monitoring has been done through the isotopic sewage plume mapping of aquatic plants in South Africa, despite its strong track record for determination of the extent of sewage influence and it potential for the early indication of eutrophication.

4.0 Stable Isotopes in Nutrient Monitoring: An N-Source Determination (Aims 1 & 2)

4.1 Introduction

The initial investigation towards assessing the feasibility of using primary producers to map anthropogenic nutrient loads in South African river systems attempted to focus on comparing stable isotope signatures from plants found in oligotrophic and eutrophic systems. The comparison of these two types of systems will help to provide an estimation of typical isotopic ranges for pristine and impacted rivers and comparisons with nutrient data will determine how well *Eichhornia crassipes* and *Nasturtium officinale* reflect nutrient changes in the systems they inhabit.

4.2. Materials and Methods

4.2.1 Experimental Design

The systems that were chosen to test the feasibility of this technique were the New Years Dam system and the Kubusi River and Wriggleswade Dam (near Stutterheim; see Fig 4.1). These systems were chosen because the former is a moderately healthy, relatively oligotrophic system,

with no indentified direct nutrient inputs, while the later is a heavily impacted system, with large-scale diffuse agricultural inputs as well as a sewage point-source in Stutterheim.

250ml water samples and isotope samples of both *Eichhornia crassipes* and *Nasturtium officinale* were taken in triplicate (when possible) at 16 different sites within each system (6 upstream of potential pollutant source, 4 at the source, 6 downstream of potential pollutant source). Presence and abundance of both water hyacinth and watercress turned out to be extremely patchy and so six other plant species were collected opportunistically in the hopes of increasing sample numbers.

4.2.2 Nutrient Analysis

Water samples taken from all sites were analyzed by segmented flow analysis for ammonium (NH_4), nitrates (NO_3^-) and phosphorous (P) at the University of KwaZulu-Natal.

4.2.3 Isotope Analysis

All isotope samples were collected fresh new growth (where possible), rinsed in distilled water and oven dried (50ºC for 24 hours). $\delta^{13}C$ and $\delta^{15}N$ signatures of all the samples were determined using either a Europa Scientific Integra IRMS or Europa Scientific 20-20 IRMS liked to an ANCA SL Elemental Analyser at the IsoEnvironmental Laboratory at Rhodes University. Beet sugar and ammonium sulphate were used as internal standards, calibrated against International Atomic Energy reference materials (PeeDee Belemnite and air for $\delta^{13}C$ and $\delta^{15}N$, respectively). Results are expressed in standard delta notation, $\delta X = ([Rsample/Rstandard] - 1) \times 1000$, 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 carbon and nitrogen was ±0.05%.

4.3 Results

Nutrient results from New Years Dam system are presented in Figure 4.2. Low levels of nutrients characterized the ecosystem upstream of the impoundment, while water samples from the dam itself had high levels of nitrates. Downstream of the dam (the golf course) however, the N source shifted, with increasing concentrations seen in ammonium. The nutrient results from the Kubusi River are more complicated, with extremely high variability, and due to the large numbers of tributaries and meanders it was not possible to put the data into any kind of stream order. As there were no discernable patterns the data are not presented here.

A number of different sampling sites were designated in both systems (Fig 4.1); however sampling was hindered by extreme patchiness in plant distributions for both *Eichhornia crassipes* and *Nasturtium officinale* and neither plant was found across an entire nutrient gradient. In addition, at some sites both plant species were rooted in sediment and this change in metabolic pathway was manifested in the isotope signatures by extremely high variation in both carbon and nitrogen. Isotopic comparisons between these plants and the other six species collected opportunistically were nonsensical, and offered no insights into unraveling nutrient dynamics in either system and are consequently not presented here.

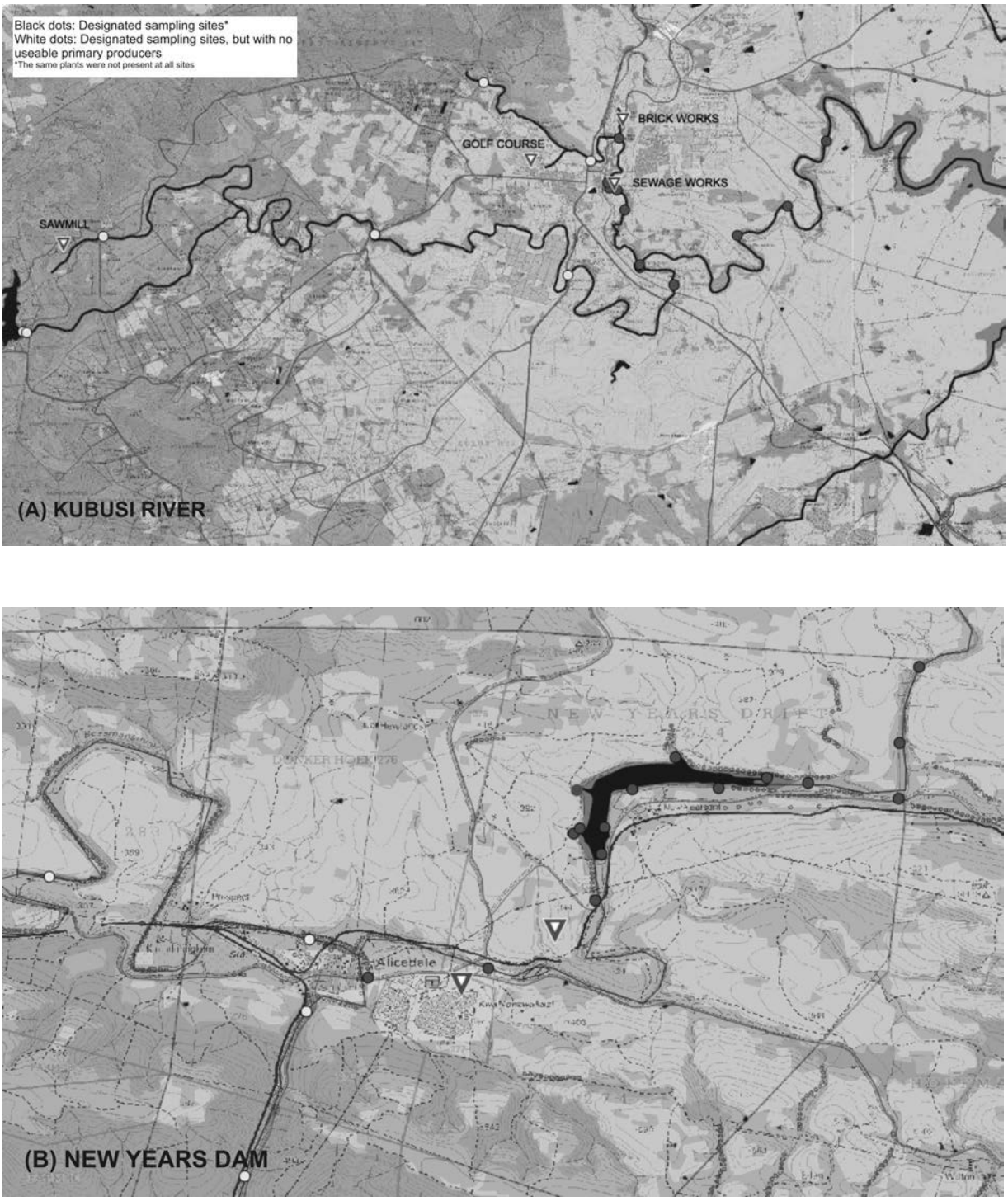

Figure 4. 1: Sampling maps for the Kubusi River system (A) and New Years Dam (B). Black dots indicate designated sample sites where nutrients and plants were collected (when possible)* and white dots indicate designated sample sites were no appropriate plants could be found. *Different plant species were collected at different sites, as plant cover was extremely patchy.

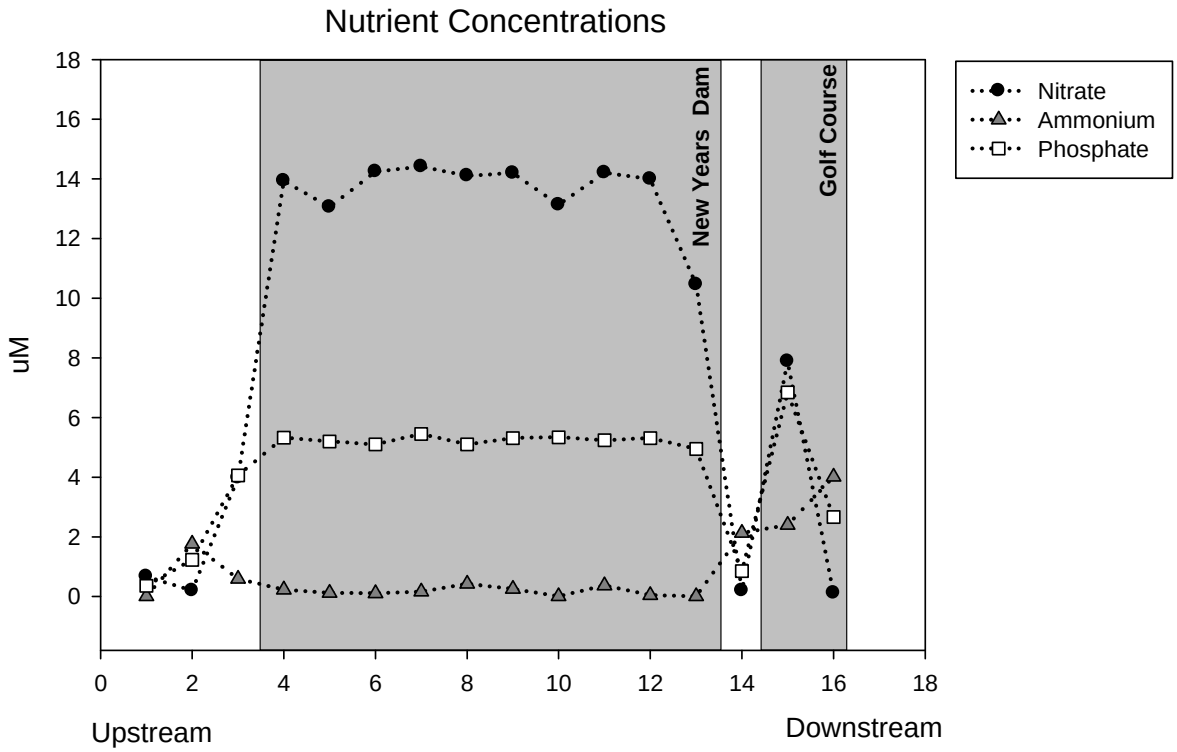

Figure 4. 2: Nutrient concentrations of water samples taken from the New Years Dam system. Neither *Eichhornia crassipes* nor *Nasturtium officinale* were found at all sites, and neither plant was found across the entire nutrient gradient.

4.4 Discussion

Nutrient analysis from the New Years Dam system (Fig 4.2) showed distinct differences in nutrient availability above, below and within the dam. This type of nutrient gradient is ideal for testing the ability of a primary producer to reflect differences in a natural environment. Unfortunately, after exhaustive field investigations it became apparent that neither *Eichhornia crassipes* nor *Nasturtium officinale* was present at sufficient sample sites to serve as a useful indicator species. In the New Years Dam system for example, although there were plenty of plants found downstream and within the dam, there were no individuals found upstream of the dam and consequently a comparison of isotopic signatures across nutrient gradients was not possible. The presence of *Nasturtium officinale* was even more patchy, with very few plants at any one site, and again no individuals found upstream. Additionally *Nasturtium officinale*, and in some cases, *Eichhornia crassipes* had rooted into sediment (MP Hill, S Kaehler pers. obs.) at a number of sites, which alters the pathways by which nutrients are acquired. Indeed the nutrients pulled up from sediments are unlikely to be indicative of water quality and/or anthropogenic nitrogen loading in the water column, but will instead represent an accumulation of nutrients from various sources over various time periods (Kellman & Hillaire-Marcel 1998).

The Kubusi River and Wriggleswade Dam system was even more complicated, as there were numerous tributaries and meanders that both bring in and remove nutrients from the ecosystem. Nutrient data was thus extremely variable, difficult to map out in an upstream-source-downstream

order and virtually impossible to evaluate in a coherent fashion. In addition to this, the abundance of *Eichhornia crassipes* and *Nasturtium officinale* within this system was even patchier. In order to cope with this, six other plant species were collected opportunistically, in the hopes of obtaining an overview of isotopic plant signatures within a highly eutrophic environment. Isotopic results however were messy, with high variation, showing unequivocally that isotope signatures of multiple plant species are not comparable, particularly across such broad nutrient gradients.

4.5 Conclusions

The original objective defined for this experiment was to begin a field based study that monitored changes in plant isotope signatures across a nutrient gradient. It was envisioned that this would be done in both wet and dry seasons because flow regime will most certainly affect nutrient availability and that by using plants from the natural environment we could map N dynamics with minimal ecosystem interference. It became quickly apparent however that *Eichhornia crassipes* and *Nasturtium officinale* were not nearly as ubiquitous as previously thought, and that the addition of multiple other species to cope with this patchiness did little to resolve N dynamics within either system. Consequently, in order to successfully map anthropogenic sources of nutrients in natural systems, a single aquatic plant species that can be easily transplanted from the lab to the field is required. Transplantation experiments guarantee that the plant in use is of an initially known isotopic concentration and ensures that it will always be present at the sites of investigation. Once studies are complete, plants can be returned to the lab, re-acclimated and potentially re-used. Transplanting invasive species is ethically unsound, particularly when dealing with both category 1 and category 2 level plants (Conservation of Agricultural Resources Act, Act 43 of 1983, amended in 2001), and so this plant would need to be small, free-floating, easily manipulated, fast growing in various flow regimes and non-invasive in South African waterways.

Spirodella sp. meets all these requirements and is an opportunistic and cosmopolitan species that could be easily grown in the lab and then transplanted in the field. It could be floated in cages at any site and allowed to take up nutrients over a defined time period to indentify nitrogen sources and could be placed over entire nutrient gradients without difficulty. This type of monitoring requires a return to laboratory experiments to generate baseline data to which field data could then be calibrated against. This is quite clearly the way forward for pollution mapping through primary producers and the next step in understanding the mechanisms driving eutrophic systems.

5.0 Tissue Turnover Rates, Concentration Level Effects And Transplantation Studies: Defining Quantitative Indicators of Nutrient Loading In Aquatic Plants (Aims 3 & 4).

5.1 Introduction

The use of stable isotopes to investigate nitrogen loading has the potential to be an excellent technique for mapping anthropogenic pollution. However, in order for this to be a useful tool, baseline information is required to make realistic inferences between nutrient status and isotopic signatures. In any natural system the quantitative relationship between nutrient status and isotopic measurements is difficult to establish, especially when working in the field. Primarily because, while isotopic signatures provide time-integrated assessments of nutrient loading, nutrient assays done in the field are spot measurements, which can be highly variable and

represent only instantaneous moments in time. These difficulties may be overcome by generating a set of baseline data, produced through manipulations in the laboratory, and, under controlled conditions, the effects of variable nutrient concentration can then be quantified in relation to changes in isotope signatures. Additionally, through sub-sampling over time, a rate of tissue isotopic turnover can be calculated which will help to define the period of time integration represented by the plant isotope signatures.

Once baseline data exist to calibrate samples taken from the natural environment, transplantation experiments of primary producers with known isotope signatures can help to monitor current nutrient loading of a system as well as to identify sources and concentration levels of anthropogenic pollution. A rapidly growing plant should be able to generate maps of pollution hotspots within days of transplantation, providing information not only on pollution distribution in an ecosystem, but actually distinguish between different pollution sources.

Aims 3 and 4 were completed in reverse order, with the intent of generating baseline data prior to applying the technique in the field. Aim 4 was completed in two parts; the first of which determined the isotopic equilibration rates (or tissue turnover times) of water hyacinth (*Eichhornia crassipes*), watercress (*Nasturtium officinale*) and subsequently *Spirodella* sp. and the second of which quantified concentration level effects on plant isotope signatures for *Spirodella* sp. only. Aim 3 investigated the practical application of field transplantation studies.

5.2 Laboratory Manipulations (Aim 4)

5.2.1 Methods

5.2.1.1 Isotopic Equilibration Rates

5.2.1.1.1 Treatment Solutions

5.2.1.1.1a) *Eichhornia crassipes & Nasturtium officinale*

Two days prior to the start of this study, 600L of treatment solution was premixed for each of three nutrient regimes; fertilizer, cow manure and tap water (control), and stored in large, clean header tanks, covered with black tarpaulin. Solutions were premixed to avoid variation artifacts in isotopic signatures of the nutrient solutions and were covered with black tarpaulin for the duration of the experiment to prevent microalgal growth, which may also alter the isotopic values of the stock solutions. 21 healthy plants of both water hyacinth and watercress were also placed in de-chlorinated tap water for two days prior to experimental start, to cleanse the root complexes of previously acquired nutrients.

Cow manure solution was created by sieving out large particulates (e.g. grass, fibers) from fresh cow manure collected from a local dairy farm (Thorn Farm, Eastern Cape, SA) and adding it de-chlorinated tap water. Fertilizer stock solution was created using Omnia Nutriology fertilizer pellets (164g N, 164g K 32g P) ground to fine powder and added to de-chlorinated tap water. The resulting slurry and fertilizer solutions were then filtered through glass fiber filters (GFFs; 0.45μm pore size) and the filtrate was analyzed using an Aquaculture photometer (2008 Series HI 83203,

Hanna Instruments), for concentrations of nitrate (NO_3^-) and ammonia (NH_3). These results were used to calculate dilutions to obtain stock solutions of 20.0mg/L N with nitrogen being present predominantly as NO_3^- and NH_3 for fertilizer and cow manure treatment solutions respectively.

5.2.1.1.1b) *Spirodella sp*.

Two days prior to the start of this study, 12L of treatment solution was premixed for each of three nutrient regimes; fertilizer, cow manure and tap water (control) and stored in large bottles, covered with black tarpaulin, the reasons for which are described above. Approximately 400g (wet weight) of *Spirodella* spp. was also placed in de-chlorinated tap water for two days prior to experimental start, to cleanse the root complexes of previously acquired nutrients.

Cow manure solution was created as previously described to obtain a stock solution of 20.0mg/L N with nitrogen being present predominantly as ammonia (NH_3). Fertilizer stock solution was created by adding potassium nitrate (KNO_3) to de-chlorinated tap water at concentrations of 20.0mg/L N (NO_3^-). As a result of the monumental die off and lack of new growth experienced by both water hyacinth and watercress when grown in and switched to tap water in the previous experiment (see results 5.3.1 and discussion) commercial iron chelate (13% Fe, EDTA-FeNa-$3H_2O$) was added to all treatment solutions at a concentration of 11.2 mg Fe/L (Coetzee et al. 2007) to avoid nutrient uptake limitation associated with iron deficiency.

Samples of synthetic fertilizer (Omnia Nutriology), KNO_3 and fresh cow manure were also collected in triplicate.

5.2.1.1.2 Experimental Design

5.2.1.1.2a) *Eichhornia crassipes* & *Nasturtium officinale*

7 plants each of both water hyacinth *(Eichhornia crassipes*) and watercress (*Nasturtium officinale*) were placed in three 50 L tubs, containing either fertilizer solution, cow manure solution or de-chlorinated tap water (run as a control). The experiment was run in triplicate (n = 3) for all treatments, over a period of 70 days. Samples of new growth from water hyacinth and watercress were taken from each tub initially and subsequently on days 1, 2, 4, 8, 16, 24 and 32. Solutions were replaced with fresh stock from the premixed header tanks on a weekly basis to compensate for nutrient depletion.

After day 32, all tubs were emptied, cleaned and re-filled with de-chlorinated tap water, in the attempt to determine if the isotope signatures of these plants would return to original values, reflecting the lack of nutrient availability. Samples of new growth (where possible) from water hyacinth and watercress were taken from each tub initially (i.e. on day 32) and subsequently on days 33, 34, 38, 46, 54, 62 and 70.

No single plant was sampled more than twice and this study was completed within the polyurethane tunnel at Rhodes University.

5.2.1.1.2b) *Spirodella* sp.

Approximately 40g (wet weight) of *Spirodella* sp. was placed in three 2L tubs containing either fertilizer solution, cow manure solution or de-chlorinated tap water (run as a control). The experiment was run in triplicate (n = 3) for all treatments over a period of 16 days. Samples were taken from *Spirodella* initially and subsequently at 6hrs, 14hrs, 24hrs, 48hrs, 4, 6, 8, 12 and 16 days. The time series was shortened considerably in this experiment because growth rates of *Spirodella* spp. are extremely high (Henderson 2001). Additionally, no switch to tap water was performed to avoid die off and/or starvation which may have influenced isotope signatures (see results 5.3.1 and discussion). This poses no practical problems however as no freshwater system in South Africa can be considered oligotrophic to the same degree.

The experiment was run in ambient temperatures (20^{o}C to 27^{o}C) under growth lights set on a 12:12 light dark cycle.

5.2.1.1.3 Isotope Analysis

All isotope samples were collected fresh new growth (where possible), rinsed in distilled water and oven dried (50ºC for 24 hours). δ^{13}C and δ^{15}N signatures of all the samples were determined using either a Europa Scientific Integra IRMS or Europa Scientific 20-20 IRMS liked to an ANCA SL Elemental Analyser at the IsoEnvironmental Laboratory at Rhodes University. Beet sugar and ammonium sulphate were used as internal standards, calibrated against International Atomic Energy reference materials (PeeDee Belemnite and air for δ^{13}C and δ^{15}N, respectively). Results are expressed in standard delta notation, $\delta X = ([Rsample/Rstandard] - 1) \times 1000$, 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 carbon and nitrogen was ±0.05%.

5.2.1.2 Concentration Effects on Isotope Signatures of *Spirodella* sp.

5.2.1.2.1 Treatment Solutions

Two days prior to the start of this study, 12L of treatment solution was premixed for five different concentrations of fertilizer and cow manure solution and stored in large bottles, covered with black tarpaulin, the reasons for which are described above. Approximately 1.2kg (wet weight) of *Spirodella* spp. was also placed in de-chlorinated tap water for two days prior to experimental start, to cleanse the root complexes of previously acquired nutrients.

Cow manure and fertilizer stock solutions were created as described in 5.2.1.1b to obtain five different treatment concentrations: zero (0.0 mg/L), low (0.5 mg/L), medium (4.5 mg/L), high (8.5mg/L), and extremely high (20.0 mg/L) nitrogen. Nutrient levels were based on concentration scales defined by Reddy et al. (1989, 1990). Commercial iron chelate (13% Fe, EDTA-FeNa-$3H_2O$) was also added to all treatment solutions at a concentration of 11.2 mg Fe/L (Coetzee et al. 2007) to avoid nutrient uptake limitation associated with iron deficiency.

5.2.1.2.2 Experimental Design

Approximately 40g (wet weight) of *Spirodella* was placed in five 2L tubs containing five different concentrations (0.0, 0.5, 4.5, 8.5, and 20.0 mg/L N) of either fertilizer or cow manure solution. The experiment was run in triplicate (n = 3) for all treatments over a period of 16 days. Samples were taken from *Spirodella* initially and subsequently at 6hrs, 14hrs, 24hrs, 48hrs, 4, 6, 8, 12 and 16 days.

The experiment was run in ambient temperatures under growth lights set on a 12:12 light dark cycle.

5.2.1.2.3 Isotope Analysis

As described in 5.2.1.1.3

5.2.2 Results

5.2.2.1 Isotopic Equilibration Rates

Both water hyacinth and watercress showed relatively long isotopic equilibration rates, with turnover times for water hyacinth at approx. 16 and 24 days for $\delta^{15}N$ and $\delta^{13}C$ respectively and with watercress having approximately the same turnover rate of 24 days for both $\delta^{15}N$ and $\delta^{13}C$ isotopes. *Spirodella*, by comparison showed extremely high turnover rates of approx. 4 days for both $\delta^{15}N$ and $\delta^{13}C$. Variation in isotopic signatures was high however, for $\delta^{13}C$ in all species (Figs 5.1 & 5.2).

Plant $\delta^{15}N$ signatures clearly distinguished between nutrient regimes, with all species grown in fertilizer solution showing strongly depleted values (ranging between -5.0 and -10 ‰) relative to controls while species grown in cow manure showed variable responses; water hyacinth grown in manure showed signatures similar to controls (~8.0 ‰), watercress showed mildly depleted values (~ 10.0 ‰; still readily distinguishable from fertilizer signatures) relative to controls (15.0 ‰) and *Spirodella* showed strongly enriched $\delta^{15}N$ (~14.0 ‰) relative to controls (~5.0 ‰) Plant $\delta^{13}C$ signatures however were less useful in distinguishing between nutrient regimes, although *Spirodella* grown in manure showed significantly more enriched signatures than those grown in fertilizer and tap water (Figs 5.1 & 5.2).

After the change to tap water in both the water hyacinth and watercress experiments, plants in the fertilizer and manure solutions experienced massive die off, and produced little to no new growth. Additionally, control plants grew very slowly and by the end of the experiment all plants had died. This was an unforeseen complication and suggests that tap water lacks enough nutrients to support plant growth. Consequently isotope signatures after day 32 may be complicated by senescence (e.g. Benner et al. 1987, Zieman et al. 1984, Fellerhoff et al. 2003) and/or nutrient starvation (Glass et al. 2001), both of which may alter isotopic chemistry. This problem was corrected in the *Spirodella* experiment by the addition of iron chelate to all solutions, and foregoing the switch to tap water.

Consistently decreasing CN ratios and increasing % nitrogen over time clearly showed increasing plant health in all species grown in fertilizer and manure solutions, while plants grown in tap

water, showed either minimal or opposite changes, indicating physiological stress. The exceptions to this were the CN ratios and % nitrogen in the *Spirodella* experiment, which showed increased plant health for all treatment solutions including the tap water control (Figs 5.3 & 5.4). The mechanism underpinning this difference in tap water can be attributed to the iron chelate added in the *Spirodella* experiment which is attached to an EDTA (ethylenediaminetetraacetic acid) complex which in retrospect clearly supplied minimal amounts of nitrogen to the system. In highly nutrient limited environments plants will take up all available nitrogen and this likely also explains the depletion in *Spirodella* tap water $\delta^{15}N$ signatures. % carbon values are also presented (Fig 5.5) but contributed very little towards the understanding of nutrient related isotope signatures.

Isotopic signatures for synthetic fertilizers and fresh cow manure fell well within the ranges described in Table 3.2, but further studies will be needed to determine local ranges of different N sources.

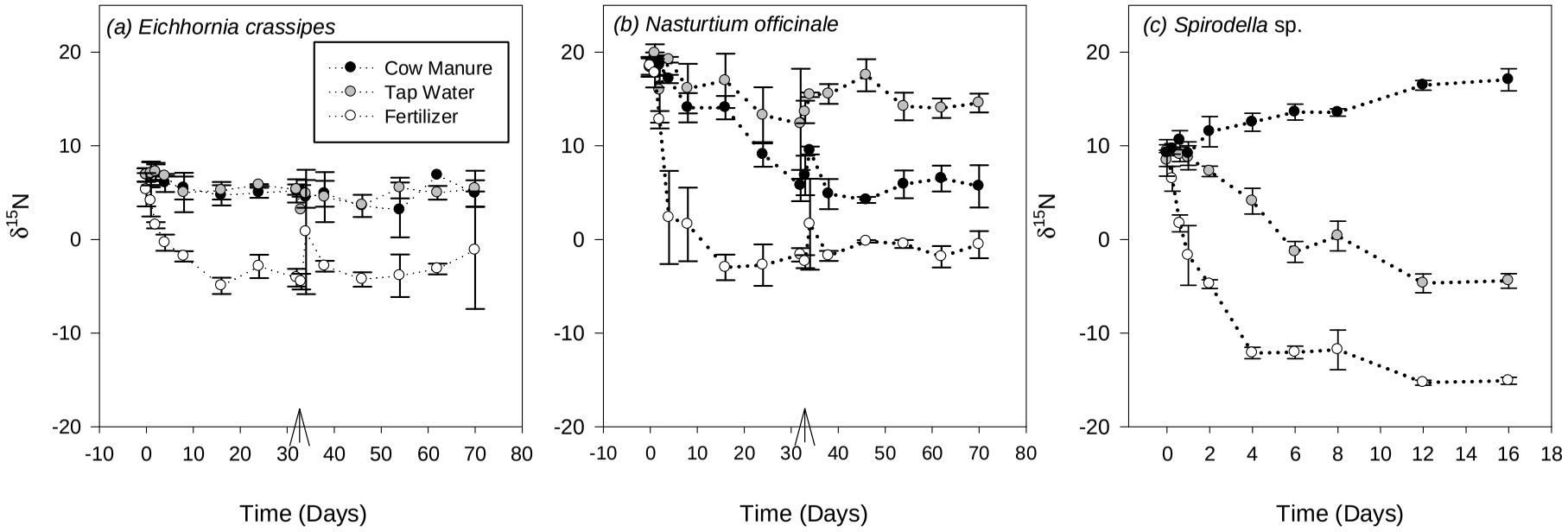

Figure 5. 1: $\delta^{15}N$ signatures of (a) *Eichhornia crassipes* (b) *Nasturtium officinale* and (c) *Spirodella* sp. growing in fertilizer, cow manure and tap water solutions. Solution concentrations were defined as extremely high (≥ 20.0 mg/L N) and tap water provided a control with a concentration of 0.0 mg/L N. The arrow in (a) and (b) indicates a switch from fertilizer/cow manure solution to tap water at day 32.

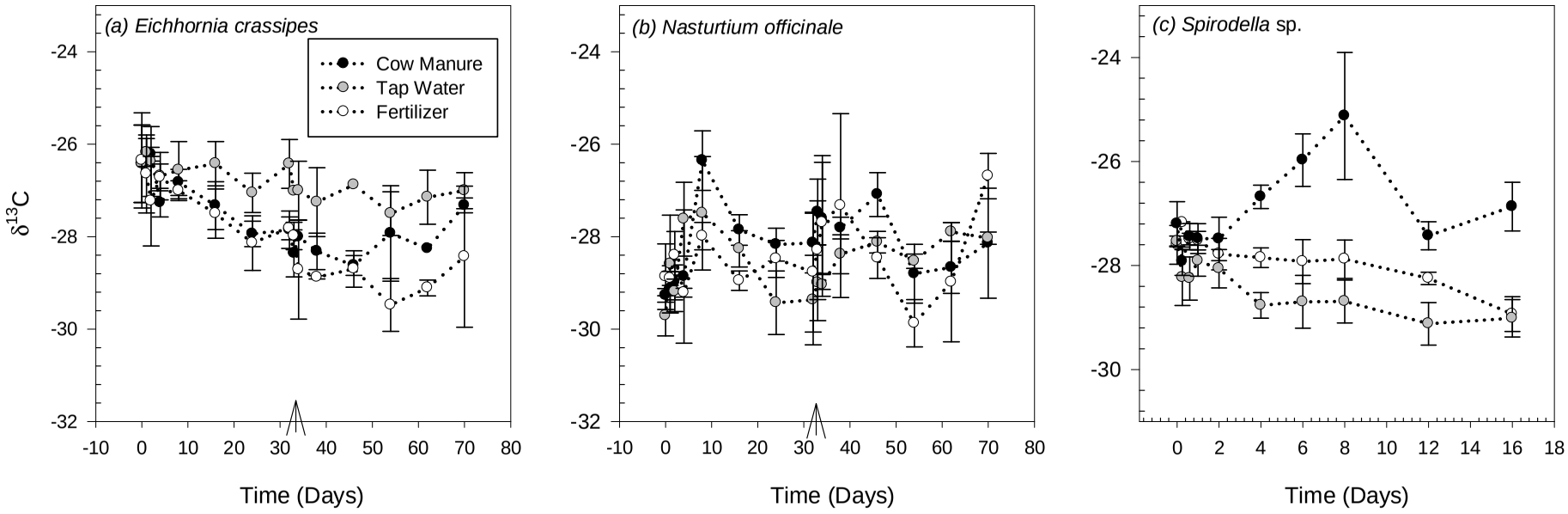

Figure 5. 2:: $\delta^{13}C$ signatures of (a) *Eichhornia crassipes* (b) *Nasturtium officinale* and (c) *Spirodella* sp. growing in fertilizer, cow manure and tap water solutions. Solution concentrations were defined as extremely high (≥ 20.0 mg/L N) and tap water provided a control with a concentration of 0.0 mg/L N. The arrow in (a) and (b) indicates a switch from fertilizer/cow manure solution to tap water at day 32.

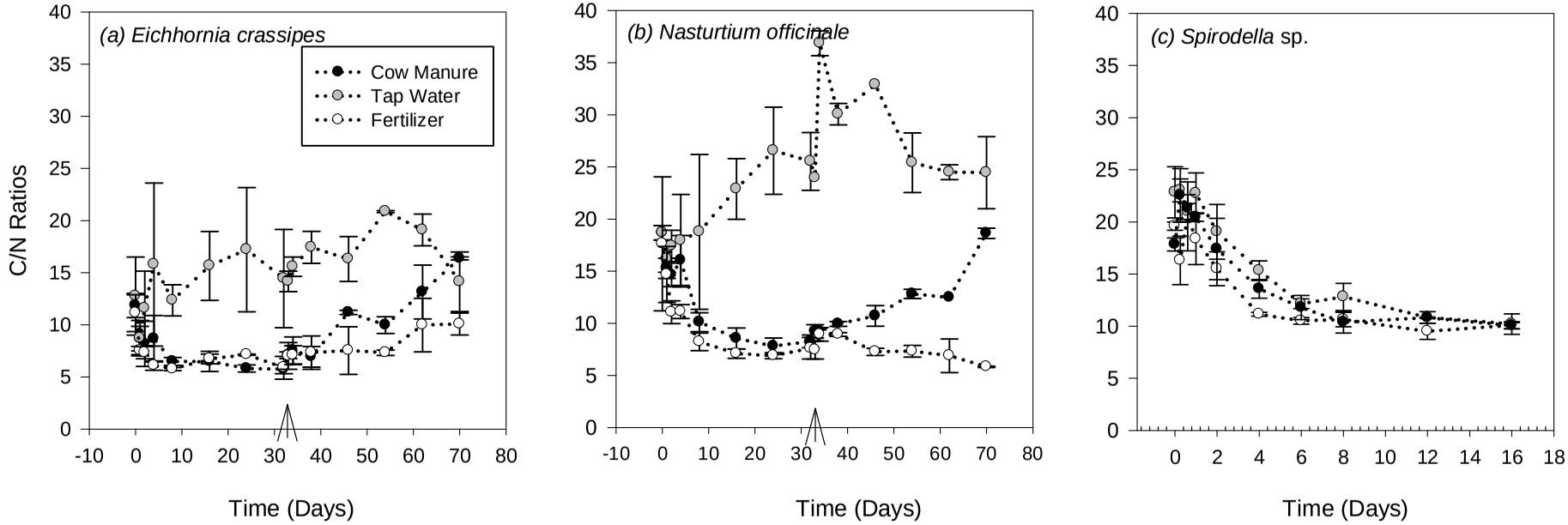

Figure 5. 3: CN Ratios of (a) *Eichhornia crassipes* (b) *Nasturtium officinale* and (c) *Spirodella* sp. growing in fertilizer, cow manure and tap water solutions. Solution concentrations were defined as extremely high (≥ 20.0 mg/L N) and tap water provided a control with a concentration of 0.0 mg/L N. The arrow in (a) and (b) indicates a switch from fertilizer/cow manure solution to tap water at day 32.

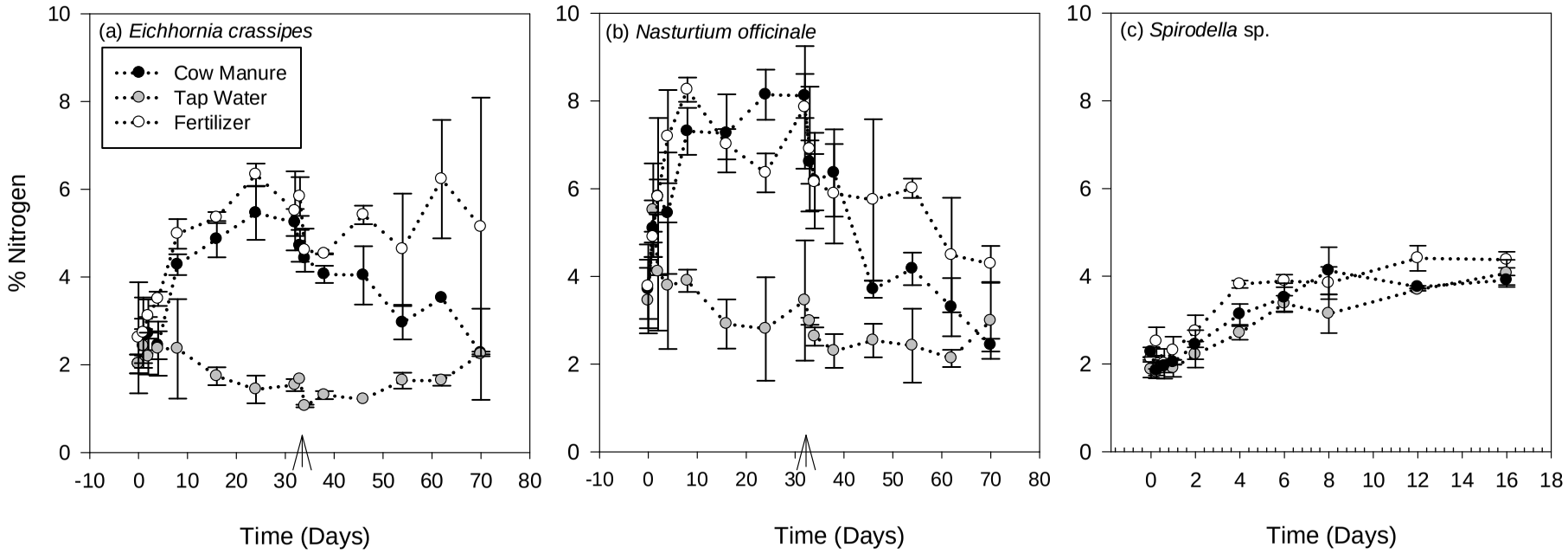

Figure 5. 4: % Nitrogen of (a) *Eichhornia crassipes* (b) *Nasturtium officinale* and (c) *Spirodella* sp. growing in fertilizer, cow manure and tap water solutions. Solution concentrations were defined as extremely high (≥ 20.0 mg/L N) and tap water provided a control with a concentration of 0.0 mg/L N. The arrow in (a) and (b) indicates a switch from fertilizer/cow manure solution to tap water at day 32.

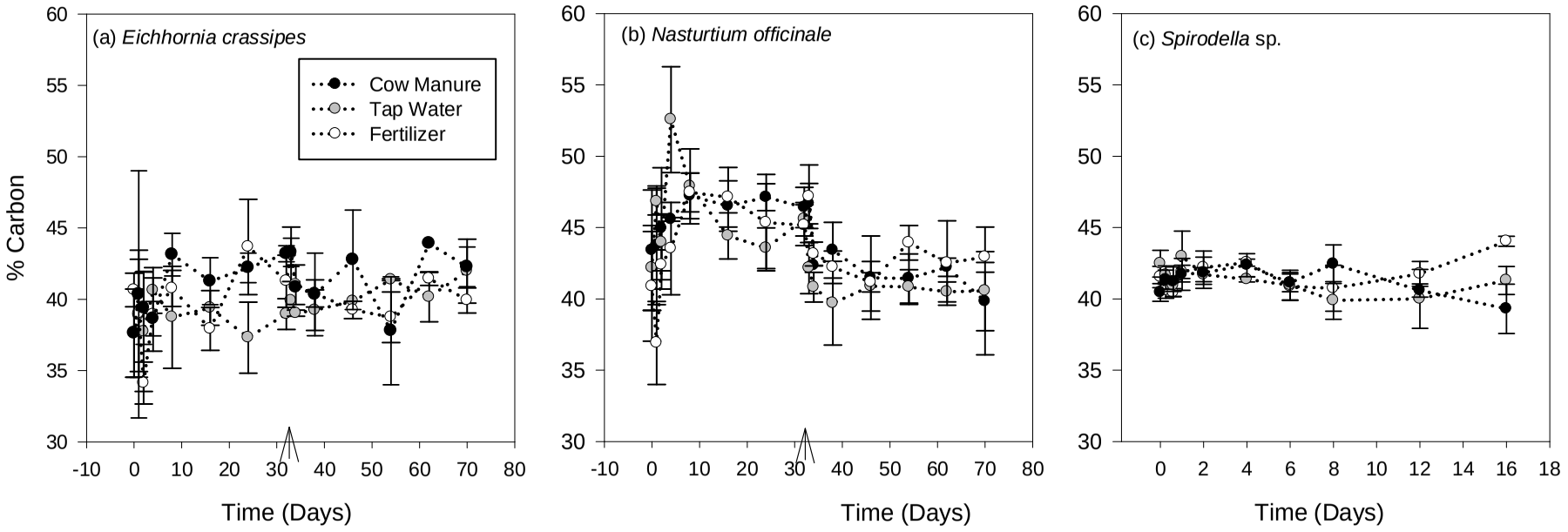

Figure 5. 5: % Carbon of (a) *Eichhornia crassipes* (b) *Nasturtium officinale* and (c) *Spirodella* sp. growing in fertilizer, cow manure and tap water solutions. Solution concentrations were defined as extremely high (≥ 20.0 mg/L N) and tap water provided a control with a concentration of 0.0 mg/L N. The arrow in (a) and (b) indicates a switch from fertilizer/cow manure solution to tap water at day 32.

5.2.2.2 Concentration Effects on Isotope Signatures of *Spirodella* sp.

Spirodella $\delta^{15}N$ signatures from plants grown in fertilizer expressed clear depletion in nitrogen values with increasing concentrations over time, while plants grown in cow manure expressed enriched $\delta^{15}N$ signatures with increasing concentration (Fig 5.6). $\delta^{13}C$ signatures were less clear, with no significant changes associated with increasing concentrations over time, variability however was higher in carbon than nitrogen, particularly for plants in high concentrations of cow manure.

Plants in both treatments, at all nutrient concentrations reflected similar increases in health over time; with CN ratios decreasing from ~23.0 initially to ~8.0 by day 16 and % nitrogen increasing from ~1.8 initially to ~4.0 by day 16 for both fertilizer and cow manure, however, no concentration level effects were apparent. Plants in zero concentration solutions once again demonstrated similar changes to those plants grown in higher nutrient levels, and again can likely be explained by the addition of iron chelate (Fig 5.7). % carbon values are also presented (Fig 5.8) but contributed very little towards the understanding of concentration level effects on isotope signatures.

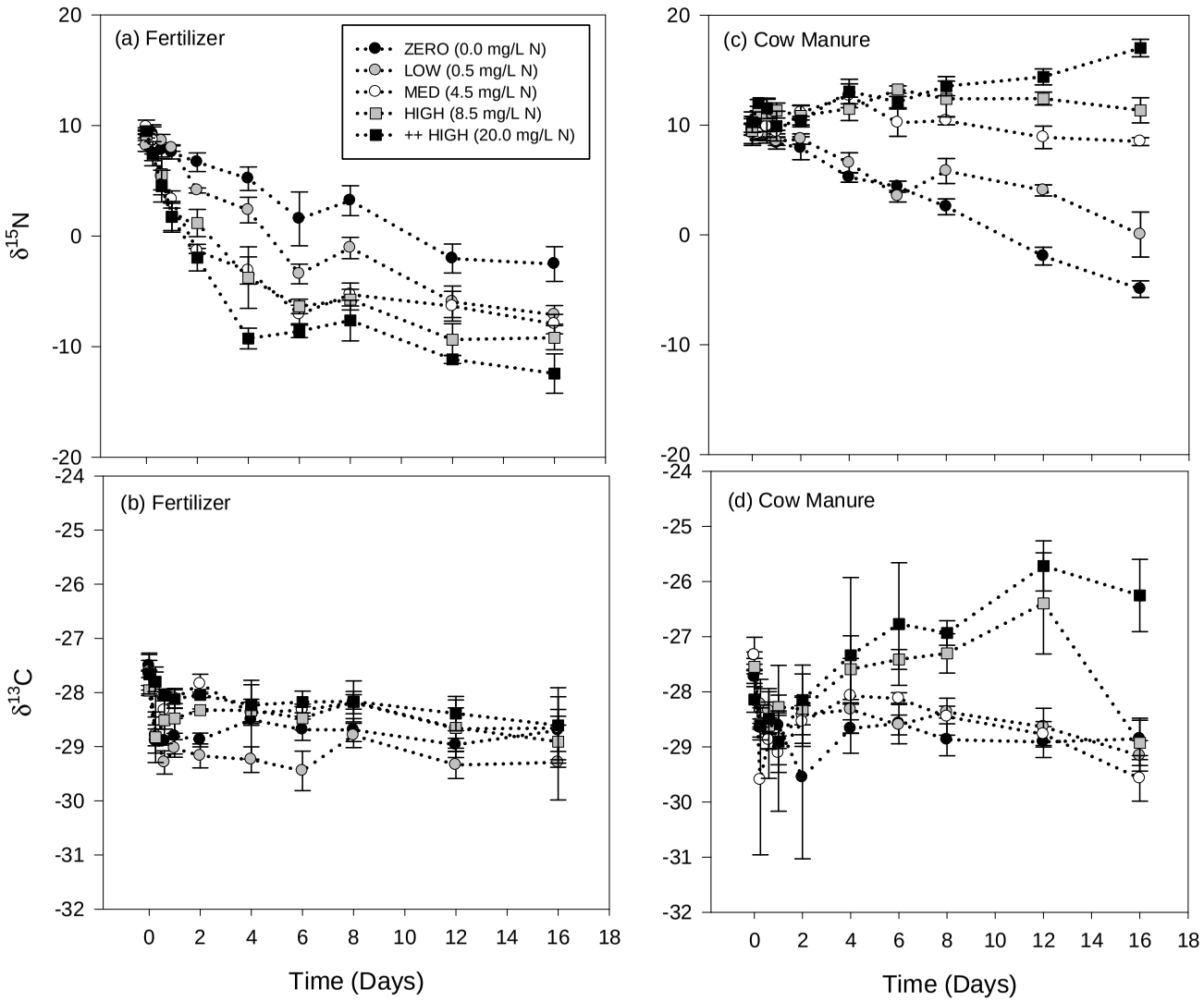

Figure 5. 6: $\delta^{15}N$ (a & c) and $\delta^{13}C$ (b & d) signatures of *Spirodella* sp. grown on fertilizer or cow manure solutions at five difference concentrations of nitrogen (mg/L N) over time.

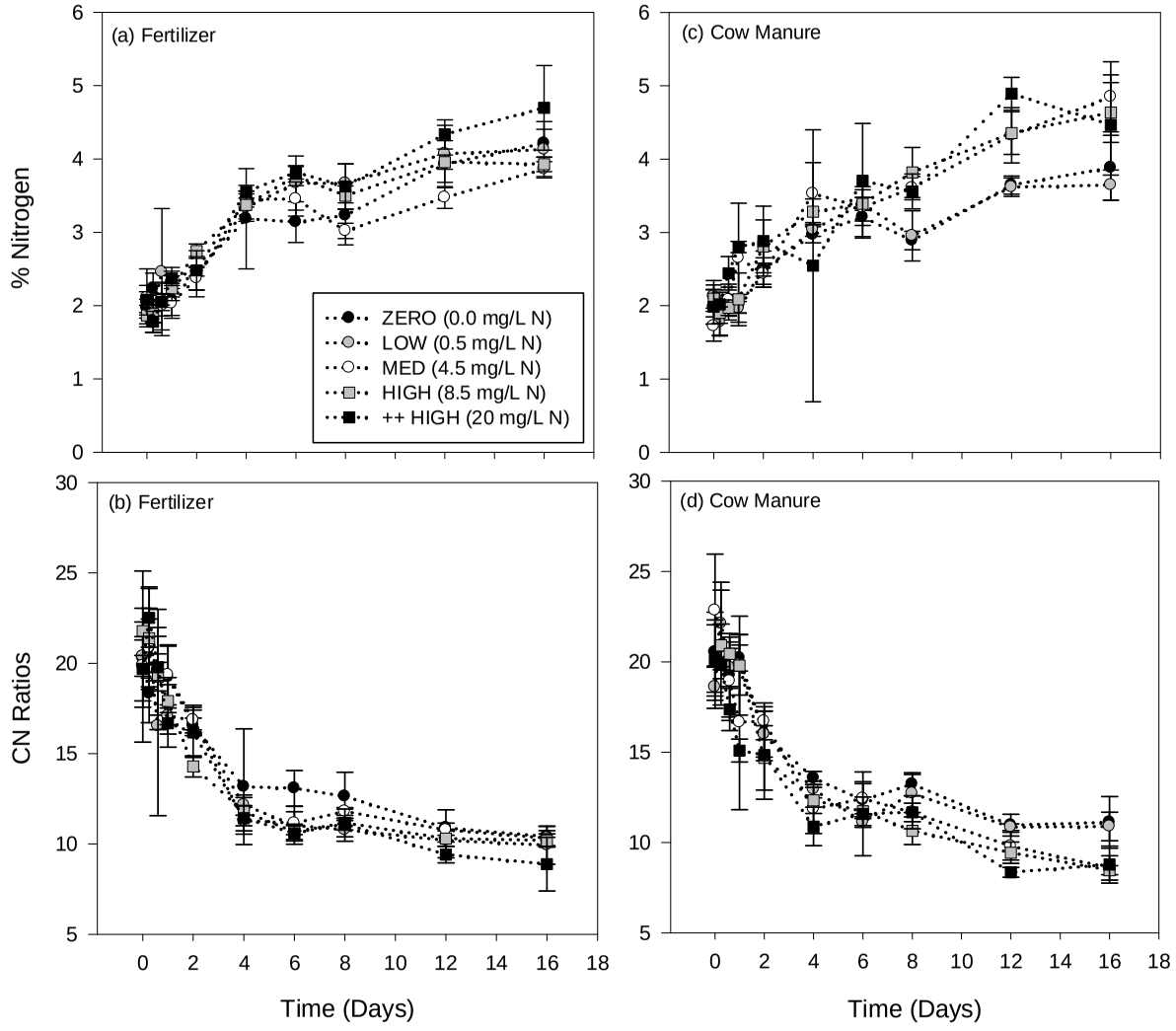

Figure 5. 7: % Nitrogen (a & c) and CN ratios (b & d) of *Spirodella* sp. grown on fertilizer or cow manure solutions at five difference concentrations of nitrogen (mg/L N) over time.

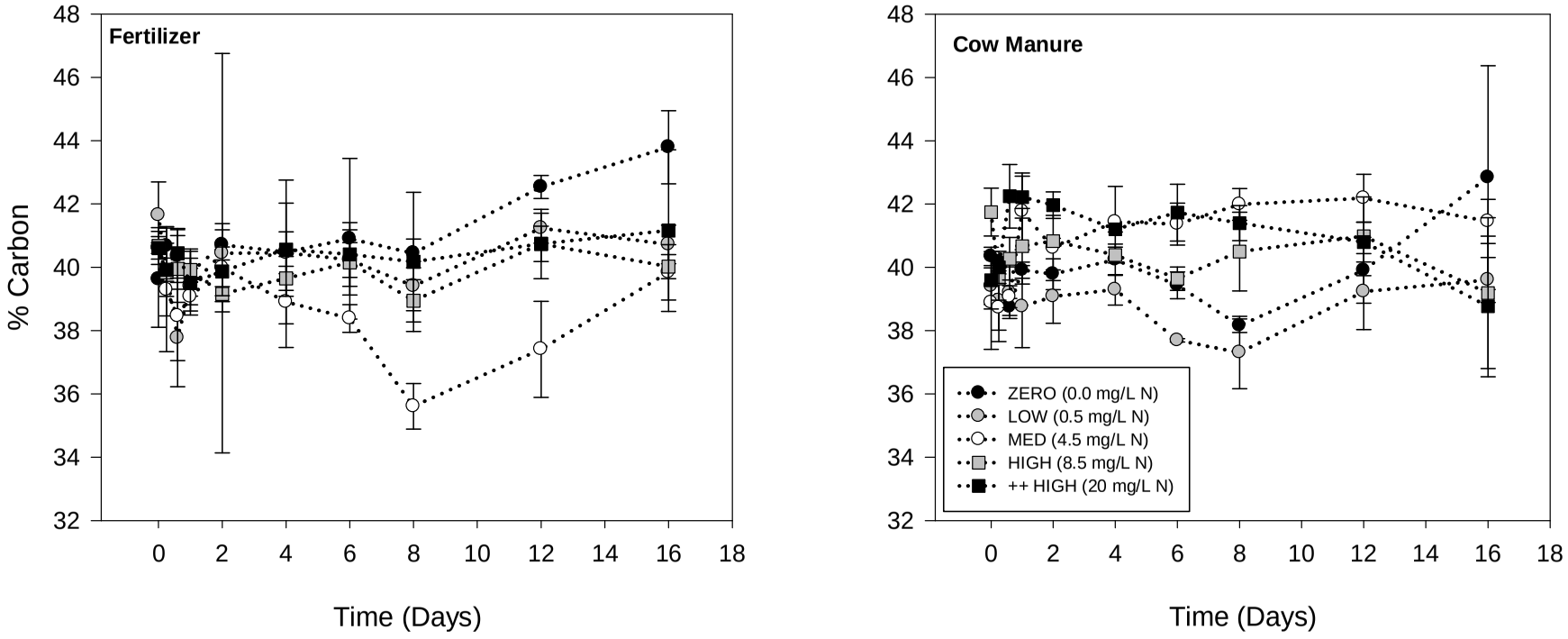

Figure 5. 8: % Carbon of *Spirodella* sp. grown on fertilizer or cow manure solutions at five difference concentrations of nitrogen (mg/L N) over time.

5.3 Transplantation Experiment (Aim 3)

5.3.1 Methods

5.3.1.1 Experimental Design

Approximately 40 g of *Spirodella* grown for 16 days on a low fertilizer concentration (0.5 mg/L N; taken from the concentration level experiment (Aim 4b)) with a $\delta^{15}N$ signature of -3.4 ± 0.89 ‰ and a $\delta^{13}C$ signature of -29.2 ± 0.24 ‰ was placed in each of 12 floating cages in a stream on small farm holding in the Eastern Cape (Brackendale Farm). The floating cages consisted of open-ended plastic cylinders approx. 16 cm high, fitted with two floats of high density foam. Floats were attached with small bolts in the middle of each cylinder, on opposite sides and the submerged section of plastic was perforated with small holes to allow water flow. Cages were designed to float freely in the water while containing the indicator species, and were prevented from drifting away by lengths of string. Six cages were placed ~500m upstream (pristine) of a complex of French drains and six were placed ~ 100m downstream (less pristine).

The experiment ran for 16 days, samples were collected initially and subsequently on days 4, 8, 10, 12 and 16. After the day 8 sample was taken, three cages from the upstream site were switched with three cages from the downstream site.

5.3.1.2 Isotope Analysis

As described in 5.2.1.1.3

5.3.2 Results

$\delta^{15}N$ signatures increased over time, but with no clear separation between upstream, or downstream values. Surprisingly however, in spite of high levels of variation, after 16 days $\delta^{13}C$ signatures of *Spirodella* reflected differences between upstream and downstream sites, with plants growing upstream showing enriched values (~ -29.5 ‰) relative to downstream plants (~ -31.5‰). These differences were also reflected in the switched plants, with *Spirodella* from upstream expressing depletions after the move to the downstream site and vice versa (Fig 5.9).

CN ratios and % nitrogen values also reflected differences in nutrient loading. After 16 days, plants grown downstream of the French drains possessed lower CN ratios and higher % nitrogen than those grown at the upstream site. These differences were also reflected in the switched plants, with *Spirodella* from upstream exhibiting lower CN ratios and higher % N after the move to the downstream site and vice versa (Fig 5.10). % carbon values are also presented (Fig 5.10) but contributed very little towards elucidating the results of transplantation experiments.

Tissue turnover rates appear to be slower in the field (approx. 8 days) which may be a reflection of slower growth owing to sub-optimal light conditions due excessive riparian vegetation and numerous cloudy days.

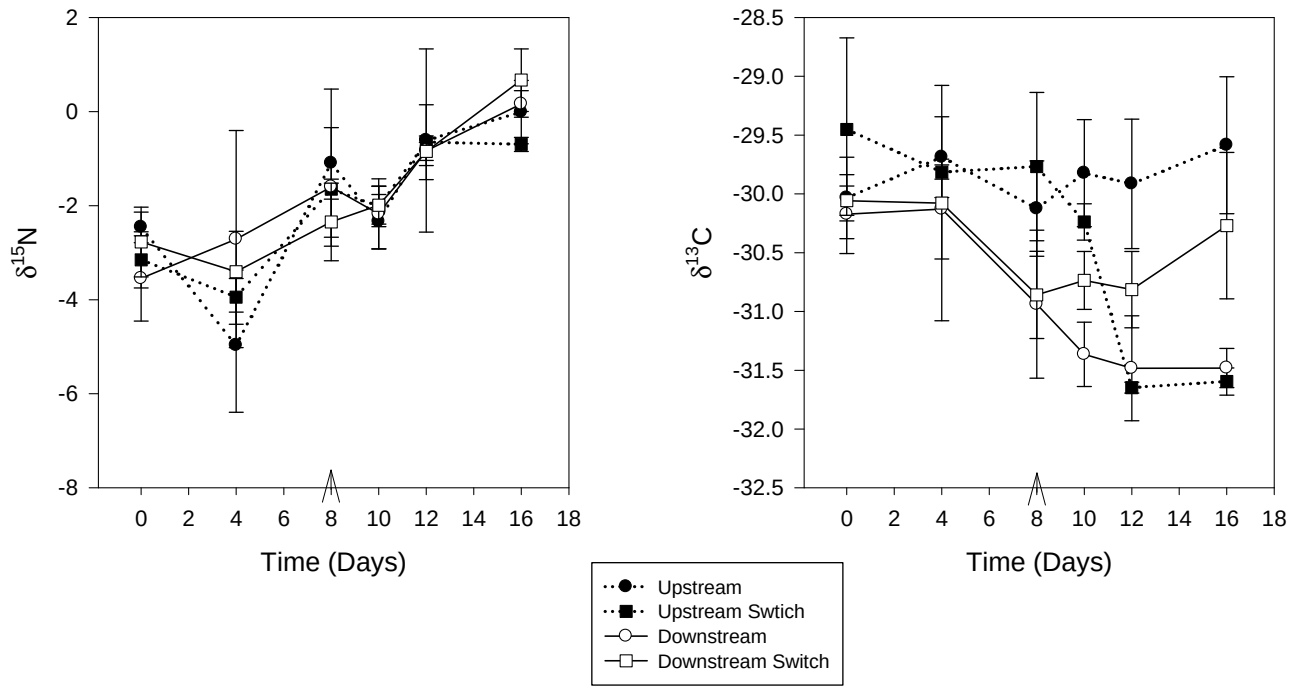

Figure 5. 9: $\delta^{15}N$ and $\delta^{13}C$ signatures of *Spirodella* sp. upstream and downstream of a complex of French drains over time. Arrow at day 8 represents a switch of three cages from upstream to downstream and vice versa. E.g. 'Upstream' refers to plants that remained upstream for the entire experiment, while 'Upstream Switch' refers to plants that started upstream and were switched to downstream after the day 8 sample collection.

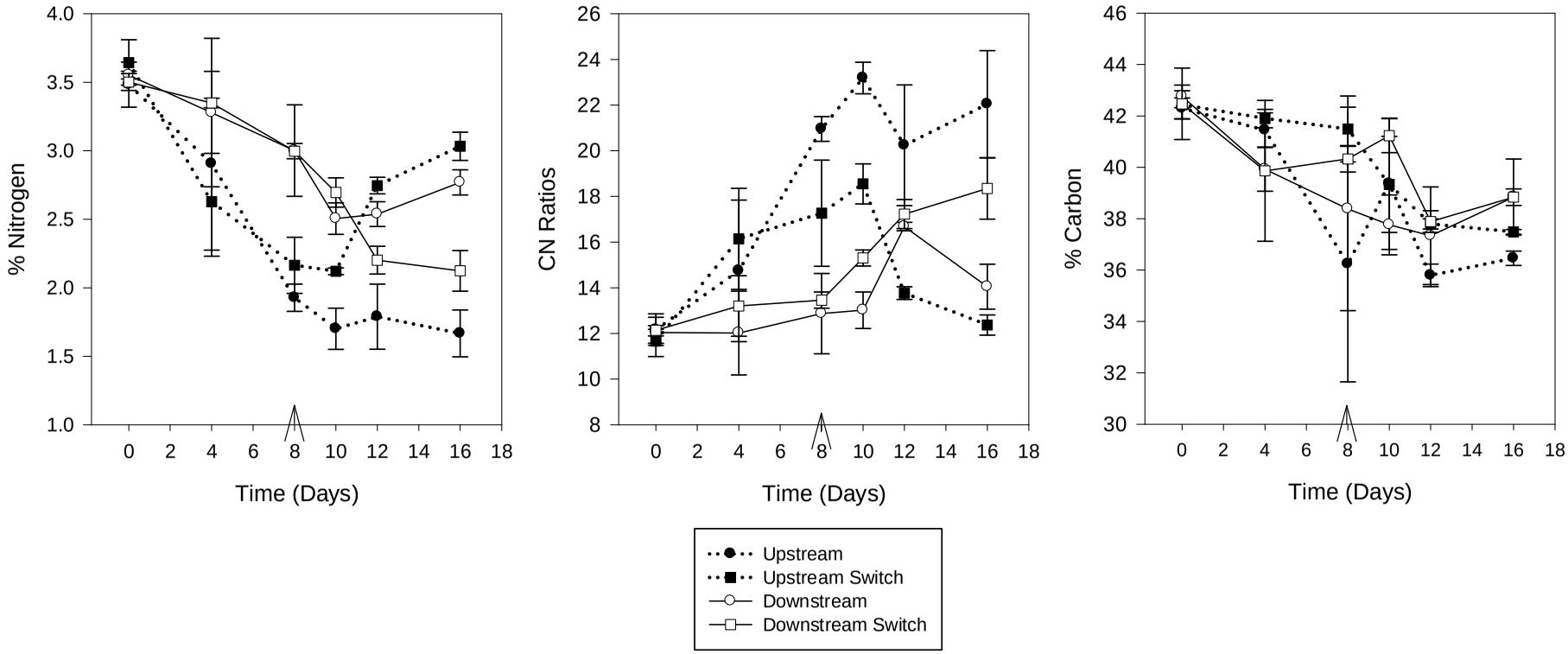

Figure 5. 10: % Nitrogen, % Carbon and CN ratios of *Spirodella* sp. upstream and downstream of a complex of French drains over time. Arrow at day 8 represents a switch of three cages from upstream to downstream and vice versa. E.g. 'Upstream' refers to plants that remained upstream for the entire experiment, while 'Upstream Switch' refers to plants that started upstream and were switched to downstream after the day 8 sample collection.

5.4 Discussion

Laboratory manipulations of water hyacinth (*Eichhornia crassipes*), watercress (*Nasturtium officinale*) and *Spirodella* sp. demonstrated that not only can δ^{15}N signatures differentiate between different sources of nutrients, but that CN ratios and % nitrogen reflect the incorporation of these nutrients into plant tissue. Plant δ^{13}C signatures however were not as useful for the differentiation of nutrient sources in the lab and % carbon added very little new information to the existing dataset. One unforeseen complication however, was the effect of iron chelate on the CN ratios and % nitrogen of the plants grown in tap water in the *Spirodella* experiment. The addition of iron chelate was an attempt to prevent iron limited nutrient uptake, which sometimes happens in oligotrophic systems (Benton Jones 2001), however in retrospect, the EDTA (ethylenediaminetetraacetic acid) complex clearly supplied minimal amounts of nitrogen to the system. In highly nutrient limited environments plants will take up all available nitrogen and this likely also explains the depletion in *Spirodella* tap water δ^{15}N signatures over time in comparison with the more or less stable values in water hyacinth and watercress.

It is important to note that the ranges of δ^{15}N and δ^{13}C signatures spanned by the different species of plants are not always the same, although they often follow similar trends. When nutrients are limiting, primary producers will take up whatever is available in the water column and isotopic fractionation is essentially zero. In unlimited systems however plants can preferentially select which isotopes they wish to assimilate and thus fractionation increases (Kendall & Doctor 2003). The amount of fractionation exhibited by a plant is species specific, as are tissue turnover times and concentration levels effects (which is why the three plant species display different δ^{15}N patterns over time in the two nutrient solutions when compared to the controls). Hence the importance of baseline datasets to calibrate the responses of the identified indicator species.

Turnover rates of water hyacinth and watercress were much longer than those of *Spirodella* and combined with the difficulties faced in finding suitable field sites that had either water hyacinth or watercress across a nutrient gradient (i.e. both above and below pollution sources), we suggest that *Spirodella* represents a better option as an indicator species. It's a small plant, possessing high growth rates and high isotopic tissue turnover, it is easily manipulated both in the lab and the field, and there no restrictions on transplantation in a natural environment. Furthermore, as a free floating plant, it avoids the problems inherent with (potentially) rooting plants (i.e. acquiring nutrients from sources other than the water column; e.g. Kellman & Hillaire-Marcel 1998).

As such, the second part of the laboratory manipulations considered only *Spirodella*, and although δ^{13}C signatures and % carbon showed no discernable patterns, δ^{15}N signatures exhibited strong, clear trends associated with increasing concentrations of both fertilizer and cow manure; fertilizer plants depleted with increasing N concentration while manure plants enriched. This concentration dependent δ^{15}N relationship signifies that these plants will not only be able to identify different sources of pollution within a riverine ecosystem, but will also be able to pinpoint pollution hotspots and generate maps of pollution distribution based on N concentrations. This confirmation of practical application is extremely exciting as this technique holds great promise for monitoring current water quality in South Africa as well as identifying variation in nutrient loading and pollution, before they effect change at the ecosystem level.

The first application of this technique in the field (using *Spirodella* sp.) provided extremely interesting data, with δ^{15}N signatures increasing over time, but with no clear separation between upstream or downstream

values. Surprisingly however, in spite of high variation, $\delta^{13}C$ signatures, CN ratios and % nitrogen values of *Spirodella* reflected differences between upstream and downstream sites, with plants growing upstream showing enriched carbon values, high CN ratios and low % N relative to those downstream. These differences were also reflected in the switched plants, with *Spirodella* from upstream expressing carbon depletions, lower CN ratios and higher %N valued after the move to the downstream site and vice versa. The lack of discernable differences in $\delta^{15}N$ signatures may be attributable to very low concentrations of N at both sites, or alternatively to differential responses to sewage – which has not yet been investigated for *Spirodella*. The former is however more likely, as reported $\delta^{15}N$ values of dissolved inorganic nitrogen (DIN) for untreated sewage ranges between 4.3-8.4 ‰ (Widory et al. 2004) and values displayed here are less than 2.0 ‰.

5.5 Conclusions

Results from these studies are incredibly promising and indicate that through the use of *Spirodella* sp. as an indicator species, nitrogen mapping, the identification of pollution hotspots and the monitoring of water quality can be done easily, in a fast and time integrated fashion as, not only do plants demonstrate concentration level isotope relationships, but transplanted individuals will reflect nutrient loading in a natural environment.

In order for this technique to reach its full potential however, further transplantation experiments need to be done across much higher nutrient gradients and on a much larger regional scale. Further research is also needed to disentangle the effects of iron chelate on isotopic plant responses, and to identify local variation in pollution source signatures.

6.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Understanding the Role of Nutrients in Aquatic Plant Invasions

Nutrient enrichment of surface water from anthropogenic (cultural) sources has long been recognized as a cause of eutrophication (Walmsley 2000). “Eutrophication” is an ecological term used to describe the process by which a body of water becomes enriched with nutrients that promote plant growth. Nutrient enrichment is often found in highly populated and developed areas where water borne sewage systems and agriculture runoff contribute to elevated loads of nutrients, particularly nitrogen and phosphorus. The increase in nutrients cause water quality and user problems as it increases and promotes the development of both living and decaying biological material (Walmsley 2000). Very few countries have escaped the problem of eutrophication and this includes South Africa, which has some of the most highly enriched surface waters in the world (Walmsley 2000). South African water bodies are enriched with phosphorus in the form of orthophosphates, polyphosphates and organic phosphates. Nitrogen is also present in many forms such as ammonium, nitrates and nitrites. These forms of phosphorus and nitrogen are all as a result of agricultural run-off and input from waste-water treatment plants (Walmsley 2000). Eutrophication causes a serious water quality problem in South Africa in terms of the increased occurrence of floating and rooted aquatic macrophytes and the first remedial step that has been taken was the promulgation of a 1mg P/l standard to be implemented into water catchment areas (Grobler 1984). This standard was introduced to reduce the quantity of orthophosphates available for plant growth so that waste water or effluent produced by, or resulting from the use of water for industrial purposes cannot contain phosphates in a higher concentration than 1 mg/l. The achievement of the phosphorus standard is however very expensive and there is a wide non-compliance with the standard (Chutter 1989).

The linkage between aquatic plant production, nutrients and human activities was first noted in the early part of this century (Walmsley 2000), whereby aquatic weed plant production is directly correlated with water nutrient concentrations particularly nitrogen and phosphorus. Thus in the presence of these water nutrients and the absence of natural enemies non-indigenous aquatic plant species are able to proliferate (Hill and Olckers 2001) posing a threat to the integrity of South African aquatic ecosystems and the quantity and quality of potable water. Control interventions are thus aimed at treating a symptom in a top-down approach and rarely address the cause, through a more strategically sound bottom-up approach. The underlying drivers of aquatic weed invasions are seldom addressed and thus the management approach has historically been reactive. Further, if the population of one aquatic weed species is reduced through a management intervention without addressing eutrophication, it is invariably replace by another weed species that is able to take advantage of the disturbed habitat.

The use of stable isotopes, as used in this study, will go a long way toward identifying and possibly quantifying the sources of pollution in rivers, lakes, dams and other impoundments, and afford water resource mangers the opportunity to address the causes of aquatic weed invasions.

6.2 Applicability of the Technique & Future Research Needs

This research was originally planned to be a field-based study. However, it soon became apparent that it was not possible to use plants in the field to obtain high resolution data and that the techniques had to be “calibrated” in the laboratory. This proved to be very successful as the data obtained from the lab

experiments showed little within-treatment variation, and we can clearly show that plants do react to different types and concentrations of nutrients. This enabled us to then apply the techniques to the field in a transplantation design which confirmed that this response (although less intense) is also apparent in the natural environment.

This technique of placing organisms into the field to monitor, in this case, changes in nutrient status of the water has rarely been used and has great potential. Transplantation as a form of monitoring still requires fine tuning, and needs to be field tested on a larger scale over a number of well defined nutrient gradients, but nonetheless holds great promise for identifying pollution sources, concentrations and distributions within aquatic ecosystems.

Stable isotope analysis has a number of different applications in the freshwater environment and could be used as a forensic tool. This technique could be used to trace the sources of pollution (as shown above) or toxins that may have caused a fish kill. Furthermore, the technique has uses in invasion biology through identifying the trophic levels that invasive organisms occupy and allowing predictions about which indigenous species they might complete with at that level. As these techniques develop it may also aid in predicting the likelihood of aquatic weed invasions and provide early warning of changes in nutrient loading before ecosystem degradation.

7.0 LITERATURE CITED

Anderson RJ, Smit AJ, Levitt GJ (1999). Upwelling and fish-factory waste as nitrogen sources for suspended cultivation of *Gracilaria gracilis* in Saldanha Bay, South Africa. *Hydrobiologia* 398/399: 455-462

Anderson C, Cabana G (2005). δ^{15}N in riverine food webs: effects of N inputs from agricultural watersheds. *Canadian Journal of Fisheries and Aquatic Sciences*. 62: 333-340

Aravena R, Evans ML, Cherry JA (1993). Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. *Ground Water* 31: 180-186

Barile PJ (2004). Evidence of anthropogenic nitrogen enrichment of the littoral waters of east central Florida. *Journal of Coastal Research*. 20: 1237-1245

Benner R, Fogel M, Sprague E, Hodson R (1987). Depletion of ^{13}C in lignin and its implications for stable carbon isotope studies. *Nature* 329: 708-710

Benson ER, O'Neil JM, Dennison WC (2008). Using the aquatic macrophyte *Vallisneria Americana* (wild celery) as a nutrient bioindicators. *Hydrobiologia*. 596:187-196

Benton Jones, J (2001). Laboratory guide for conducting soils tests and plant analysis. CRC Press, New York, pp. 191-227.

Cabana G, Rasmussen JB (1996). Comparison of aquatic food chains using nitrogen isotopes. *Proceedings of the National Academy of Science USA*. 93: 10844-10847

Carpenter SR, Bolgrien D, Lathrop RC, Stowe CA, Reed T (1998). Ecological and economic analysis of lake eutrophication by non-point source pollution. *Australian Journal of Ecology*. 23: 68-79

Castro P, Valiela I, Freitas H (2007). Eutrophication in Portuguese estuaries evidenced by δ^{15}N of macrophytes. *Marine Ecology Progress Series*. 351: 43-51

Clément J-C, Holmes RM, Peterson BJ, Pinay G (2003). Isotopic investigation of denitrification in a riparian ecosystem in western France. *Journal of Applied Ecology*. 40: 1035-1048

Coetzee JA, Byrne MJ, Hill MP (2007). Impact of nutrients and herbivory by *Eccritotarsus catarinensis* on the biological control of water hyacinth, *Eichhornia crassipes*. *Aquatic Botany* 86:179-186

Cole ML, Valiela I, Kroeger KD, Tomasky GL, Cebrian J, Wigand C, McKinney RA, Grady SP, Carvalho da Silva MH (2004). Assessment of a δ^{15}N isotopic method to indicate anthropogenic eutrophication in aquatic ecosystems. *Journal of Environmental Quality*. 33: 124-132

Costanzo SD, O'Donohue MJ, Dennison WC (2004). Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. *Marine Pollution Bulletin* 48: 514-525

Costanzo SD, O'Donohue MJ, Dennison WC, Loneragan NR, Thomas M (2001). A new approach for detecting and mapping sewage impacts. *Marine Pollution Bulletin*. 42: 149-56

Costanzo SD, Udy J, Longstaff B, Jones A (2005). Using nitrogen stable isotope ratios ($\delta^{15}N$) of macroalgae to determine the effectiveness of sewage upgrades: changes in the extent of sewage plumes over four years in Moreton Bay, Australia. *Marine Pollution Bulletin* 51: 212-217

Curt MD, Aguado P, Sanchez G, Bigeriego M & Fernandez J (2004). Nitrogen isotope ratios of synthetic and organic sources of nitrate water contamination in Spain. *Water, Air, and Soil Pollution*. 151: 135-142

Dhargalkar V (1986). Effect of treated sewage on growth of marine algae. *Indian Journal of Marine Science*. 15: 33-36

De Bruyn AMH, Marcogliese DJ, Rasmussen JB (2003). The role of sewage in a large river food web. *Canadian Journal of Fisheries and Aquatic Sciences*. 60: 1332-1344

De Bruyn AMH, Rasmussen JB (2002). Quantifying assimilation of sewage-derived organic matter by riverine benthos. Ecological Applications. 12: 511-520

Deegan LA, Wright A, Ayvazian SG, Finn JT, Golden H, Merson RR, Harrison J (2002). Nitrogen loading alters seagrass ecosystem structure and support of higher trophic levels. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 12: 193-212

Deutsch B, Voss M (2006). Anthropogenic nitrogen input traced by means of delta ^{15}N values in macroalgae: results from in-situ incubation experiments. *Science of the Total Environment*. 366: 799-808

Düreth S, Herrmann R, Pecher K (1986). Tracing faecal pollution by coprostanol and intestinal bacteria in an ice-covered Finnish lake loaded with both industrial and domestic sewage. 28: 131-149

Fellerhoff C, Voss M, Wantzen K (2003). Stable carbon and nitrogen isotope signatures of decomposing tropical macrophytes. *Aquatic Ecology* 37: 361-375

Fong P, Boyera KE, Zedler JB (1998). Developing an indicator of nutrient enrichment in coastal estuaries and lagoons using tissue nitrogen content of the opportunistic alga, *Enteromorpha intestinalis* (L. Link). *Journal of Experimental Marine Biology and Ecology*. 231: 63-79

Fourqurean JW, Moore TO, Fry BJ, Hollibaugh JT (1997). Spatial and temporal variation in C:N:P ratios, $\delta^{15}N$ and $\delta^{13}C$ of eelgrass *Zostera marina* as indicators of ecosystem processes, Tomales Bay, California, USA. *Marine Ecology Progress Series*. 157: 147-157

Fry B (1999). Using stable isotopes to monitor watershed influences on aquatic trophodynamics. *Canadian Journal of Fisheries and Aquatic Sciences*. 56: 2167-2171

Fry B, Allen YC (2003). Stable isotopes in zebra mussels as bioindicators of river-watershed linkages. *River Research and Applications*. 19: 683-696

Galloway JN, Howarth RW, Michaels AF, Nixon SW, Prospero JM, Dentener FJ (1996). Nitrogen and phosphorus budgets of the North Atlantic Ocean and its watershed. *Biogeochemistry*. 35: 3-25

Gartner A, Lavery P, Smit AJ (2002). Use of $\delta^{15}N$ signatures of different functional forms of macroalgae and filter-feeders to reveal temporal and spatial patterns in sewage disposal. *Marine Ecology Progress Series*. 235: 63-73

Gaston TF, Suthers IM (2004). Spatial variation in $\delta^{13}C$ and $\delta^{15}N$ of liver, muscle and bone in a rocky reef planktivorous fish: the relative contribution of sewage. Journal of Experimental Marine Biology and Ecology 304: 17-33

Glass ADM, Britto DT, Kaiser BN, Kronzucker HJ, Kumar A, Okamoto M, Rawat SR, Siddiqi MY, Silim SM, Vidmar JJ, Zhuo D (2001). Nitrogen transport in plants, with emphasis on the regulation of fluxes to match plant demand. *Pflanzenernähr. Bodenkd*. 164: 199-207

Grobler, D.C. (1984). Impact of eutrophication control measures on the trophic status of South African Impoundments. WRC Report No. 130/1/84.

Green EK, Galatowitsch SM (2002.) Effects of *Phalaris arundinacea* and nitrate-N addition on the establishment of wetland plant communities. *Journal of Applied Ecology*. 39: 134-144

Grice AM, Loneragan NR, Dennison WC (1996). Light intensity and the interactions between physiology, morphology and stable isotope ratios in five species of seagrass. *Journal of Experimental Marine Biology and Ecology*. 195: 91-110

Handley L, Raven J (1992). The use of natural abundance of nitrogen isotopes in plant physiology and ecology. *Plant, Cell and Environment*. 15: 965-985

Hansson S, Hobbie JE, Elmgren R, Larsson U, Fry B, Johansson S (1997). The stable nitrogen isotope ratio as a marker of food-web interactions and fish migration. *Ecology*. 78: 2249-2257

Harrington RR, Kennedy BP, Chamberlain CP, Blum JD, Folt CL (1998). ^{15}N enrichment in agricultural catchments: field patterns and applications to tracking Atlantic Salmon (*Salmo salar*). *Chemical Geoogy.* 147: 281-294

Heaton THE (1986). Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: A review. *Chemical Geology* 59: 87-102

Hebert CG, Wassenaar LI (2001). Stable nitrogen isotopes in waterfowl feathers reflect agricultural land use in western Canada, *Environmental Science and Technology* 35: 3482-3487

Henderson L (2001). Alien weeds and invasive plants: a complete guide to declared weeds and invaders in South Africa. Agricultural Research Council, Plant Protection Research Institute, Pretoria 300pp

Hill MP, Olckers T (2000). Biological control initiatives against water hyacinth in South Africa: constraining factors, success and new courses of action. In: Julian MH, Centre TD, Hill MP (Eds.), Proceedings of the Second Global Working Group Meeting for the Biological and Integrated Control Water Hyacinth, October 9-12, 2000, Beijing, China

Hobbie JE, Larsson U, Elmgren R, Fry B (1990). Sewage derived ^{15}N in the Baltic traced in *Fucus*. EOS 71: 190.

Hodgkiss IJ, Ho KC (1997). Are changes in N: P ratios in coastal waters the key to increased red tide blooms? *Hydrobiologia*. 352:141-147

Howarth RW (1998). An assessment of human influences on fluxes of nitrogen from the terrestrial landscape to the estuaries and continental shelves of the North Atlantic Ocean. *Nutrient Cycling in Agroecosystems*. 52: 213-223

Hudson N, Bakera A, Reynolds D (2007). Fluorescence analysis of dissolved organic matter in natural, waste and polluted waters — a review. *River Research and Applications*. 23: 631-649

Jones AB, O'Donohue MJ, Udy J, Dennison WC (2001). Assessing ecological impacts of shrimp and sewage effluent: biological indicators with standard water quality analyses. *Estuarine, Coastal and Shelf Science*. 52: 91-109

Jordan MJ, Nadelhoffer KJ, Fry B (1997). Nitrogen cycling in forest and grass ecosystems irrigated with ^{15}N-enriched wastewater. *Ecological Applications*. 7: 864-881

Kellman L, Hillaire-Marcel C (1998). Nitrate cycling in streams: using natural abundances of NO_3-δ^{15}N to measure in situ denitrification. *Biogeochemistry*. 43: 273-292

Kendall C (1998). Tracing nitrogen sources and cycling in catchments. *In*: C Kendall, JJ McDonnell (eds) Isotope tracers in catchment hydrology. Elsevier Science B.V., Amsterdam. pp. 519-576

Kendall C & Doctor DH (2003). Stable isotope applications in hydrologic studies. *Treatise on Geochemistry*. 5: 319-364

Kohl DH, Shearer GB, Commer B (1971). Fertilizer nitrogen: contribution to nitrate in surface water in a corn belt watershed. *Science*. 174: 1331-1334

Kreitler CW, Browning LA (1983). Nitrogen-isotope analysis of groundwater nitrate in carbonate aquifers: natural sources versus human pollution. *Journal of Hydrology* (Amsterdam). 61: 285-301

Kreitler CW, Ragone SE, Katz BG (1978). ^{15}N/^{14}N ratios of ground-water nitrate, Long Island, New York. *Ground Water*. 16: 404-409

Lagerwerff JV, Biersdorf GT, Brower DL (1976). Retention of Metals in Sewage Sludge II: Incorporated Radioisotopes. *Journal of Environmental Quality*. 5: 23-25

Lake JL, McKinney RA, Osterman FA, Pruell RJ, Kiddon J, Ryba SA, Libby AD (2001). Stable nitrogen isotopes as indicators of anthropogenic activities in small freshwater systems. *Canadian Journal of Fisheries and Aquatic Sciences*. 58: 870-878

Lapointe BE (1997). Nutrient thresholds for bottom-up control of macroalgal blooms on coral reefs in Jamaica and southeast Florida. *Limnology and Oceanography*. 42: 1119-1131

Lee V, Olsen S (1985). Eutrophication and management initiatives for the control of nutrient inputs to Rhode Island coastal lagoons. *Estuaries* 8: 191-202

Lin H-J, Wu C-Y, Kao S-J, Kao W-Y, Meng P-J (2007). Mapping anthropogenic nitrogen through point sources in coral reefs using $\delta^{15}N$ in macroalgae. *Marine Ecology Progress Series*. 335: 95-109

Lindau CW, Delaune RD, Patrick WH Jr., Lambremont EN (1989). Assessment of stable nitrogen isotopes in fingerprinting surface water inorganic nitrogen sources. *Water, Air and Soil Pollution* 48: 489-496

Lyngby J, Mortensen M (1994). Assessment of nutrient availability and limitation using macroalgae. *Journal of Aquatic Ecosystem Health*. 3: 27-34

Macko SA, Ostrom NE (1994). Pollution studies using stable isotopes. *In* Lajtha K, Michener R (eds) Stable isotopes in ecology and environmental science. Blackwell Scientific Publ., Oxford, pp 45-62.

Martinetto P, Teichberg M, Valiela I (2006). Coupling of estuarine benthic and pelagic food webs to land-derived nitrogen sources in Walquoit Bay, Massachusetts, USA. *Marine Ecology Progress Series*. 307: 37-48

Mayer B, Boyer EW, Goodale C, Jaworski NA, Van Breemen N, Howarth RW, Seitzinger S, Billen G, Lajtha K, Nadelhoffer K, Van Dam D, Hetling LJ, Nosall M, Paustian K (2002). Sources of nitrate in rivers draining sixteen watersheds in the northeastern US.: isotopic constraints. *Biogeochemistry*. 57/58: 171-197

McCarthy LH, Robertson K, Hesslein RH, Williams TG (1997). Baseline studies in the Slave River, NWT, 1990- 1994: Part IV. Evaluation of benthic invertebrate populations and stable isotope analyses. *The Science of the Total Environment*. 197: 111-125

McClelland JW, Valiela I (1998a). Linking nitrogen in estuarine producers to land-derived sources. *Limnology and Oceanography*. 43: 577-585

McClelland JW, Valiela I (1998b). Changes in food web structure under the influence of increased anthropogenic nitrogen inputs to estuaries. *Marine Ecology Progress Series*. 168: 259-271

McClelland JW, Valiela I, Michener RH (1997). Nitrogen-stable isotope signatures in estuarine food webs: a record of increasing urbanization in coastal watersheds. *Limnology and Oceanography*. 42: 930-937

McKinney RA, Nelson WG, Wigand C, Charpentier MA (2001). Ribbed mussel nitrogen isotope signatures reflect nitrogen sources in coastal salt marshes. *Ecological Applications*. 11: 203-214

Middelburg JJ, Nieuwenhuize J (2001). Nitrogen isotope tracing of dissolved inorganic nitrogen behaviour in tidal estuaries. *Estuarine, Coastal and Shelf Science*. 53: 385-391

Mitsch WJ, Day JW Jr, Gilliam JW, Groffman PM, Hey DL, Randall GW, Naiming W (2001) Reducing nitrogen loading to the Gulf of Mexico from the Mississippi River basin: strategies to counter a persistent ecological problem. *Bioscience*. 51: 373-388

Monteiro PMS, Anderson RJ, Woodbourne S (1997). $\delta^{15}N$ as a tool to demonstrate the contribution of fish-waste-derived nitrogen to an *Ulva* bloom in Saldanha Bay, South Africa. *South African Journal of Marine Science* 18: 1-9

Moore SK, Suthers IM (2005). Can the nitrogen and carbon stable isotopes of the pygmy mussel, *Xenostrobus securis*, indicate catchment disturbance for estuaries in northern New South Wales, Australia? *Estuaries* 28: 714-725

Morrison G, Fatoki OS, Zinn E, Jacobsson D (2000). Sustainable development indicators for urban water systems: A case study evaluation of King William's Town, South Africa, and the applied indicators. *Water SA*. 27: 219-232

Nixon SW (1995). Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia*. 41: 199-219

Nixon SW, Oviatt CA, Frithsen J, Sullivan B (1986). Nutrients and the productivity of estuarine and coastal marine ecosystems. *Journal of the Limnology Society of South Africa*.12: 43-71

Owens NJP (1987). Natural variations in ^{15}N in the marine environment. *Advances in Marine Biology*. 24: 389-451

Piola RF, Moore SK, Suthers IM (2006). Carbon and nitrogen stable isotope analysis of three types of oyster tissue in an impacted estuary. *Estuarine, Coastal and Shelf Science*. 66: 255-266

Pruell, RJ, Taplin BK, Lake JL, Jayaraman S (2006). Nitrogen isotope ratios in estuarine biota collected along a nutrient gradient in Narragansett Bay, Rhode Island, USA. *Marine Pollution Bulletin*. 52: 612-620

Rabalais N (2002) Nitrogen in aquatic ecosystems. *Ambio*. 31: 102-112

Rau GH, Sweeney RH, Kaplang IR, Mearns AJ, Young DR (1981) Differences in animal ^{13}C, ^{15}N and D abundance between a polluted and an unpolluted coastal site: likely indicators of sewage uptake by a marine food web. *Estuarine, Coastal and Shelf* Science. 13: 700-707

Reddy KR, Agami M, Tucker JC (1989). Influence of nitrogen supply rates on growth and nutrient storage by water hyacinth (*Eichhornia crassipes*) plants. *Aquatic Botany* 36: 33-43

Reddy KR, Agami M, Tucker JC (1990). Influence of phosphorus supply on growth and nutrient storage by water hyacinth (*Eichhornia crassipes*) plants. *Aquatic Botany* 37: 355-365

Reina M, Espina JL, Serrano L (2006). Sediment phosphate composition in relation to emergent macrophytes in the Doñana Marshes (SW Spain). *Water Research* 40: 1185-1190

Reynolds-Vargas J, Fraile-Merino J, Hirata R (2006) Trends in nitrate concentrations and determination of its origin using stable isotopes (^{18}O and ^{15}N) in groundwater of the western Central Valley, Costa Rica. *Ambio*. 35: 229-236

Roberts DE (1996). Effects of the North Head deep-water sewage outfall on nearshore coastal reef macrobenthic assemblages. *Marine Pollution Bulletin*. 33: 303-308

Rogers K (1999). Effects of sewage contamination on macro-algae and shellfish at Moa Point, New Zealand using stable carbon and nitrogen isotopes. *New Zealand Journal of Marine and Freshwater Research*. 33: 181-188

Rogers KM (2003). Stable carbon and nitrogen isotope signatures indicate recovery of marine biota from sewage pollution at Moa Point, New Zealand. *Marine Pollution Bulletin*. 46: 821-827

Savage C (2005). Tracing the influence of sewage nitrogen in a coastal ecosystem using stable nitrogen isotopes. *Ambio* 34: 145-150

Savage C, Elmgren R, Larsson U (2002). Effects of sew age-derived nutrients on an estuarine macrobenthic community. *Marine Ecology Progress Series*. 243: 67-82

Savage C, Elmgren R (2004). Macroalgal (*Fucus vesiculosus*) δ^{15}N values trace decrease in sewage influence. *Ecological Applications*. 14: 517-26

Savage C, Leavitt PR, Elmgren R (2004). Distribution and retention of effluent nitrogen in surface sediments of a coastal bay. *Limnology and Oceanography*. 49: 1503-1511

Schlacher TA, Liddell B, Gaston TF, Schlacher-Hoenlinger M (2005). Fish track wastewater pollution to estuaries. *Oecologia*. 144: 570-584

Smith VH (1998) Cultural eutrophication of inland, estuarine, and coastal waters. *In*: ML Pace, PM Groffman (eds), *Successes, Limitations, and Frontiers in Ecosystem Science*, Springer, New York, pp. 7-49

Smith-Evans M, Dawes A (1996). Early experiences in monitoring the effects of Hong Kong's new generation of sewage outfalls on the environment. *Marine Pollution Bulletin* 33**:** 317-327

Spies RB, Kruger H, Ireland R, Rice DW (1989). Stable isotope ratios and contaminant concentrations in a sewage-distorted food web. *Marine Ecology Progress Series*. 54: 157-170

Stedmon CA, Markager S, Bro R (2003). Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Marine Chemistry*. 82: 239-254.

Steffy LY, Kilham SS (2004). Elevated δ^{15}N in stream biota in areas with septic tank systems in an urban watershed. *Ecological Applications*. 14: 637-641

Stewart GR, Aidar MPM, Joly CA, Schmidt S (2002). Impact of point source pollution on nitrogen isotope signatures (δ^{15}N) of vegetation in SE Brazil. *Oecologia*. 131: 468-472

Sweeney RE, Kaplan IR (1980). Tracing flocculent industrial and domestic sewage transport on San Pedro shelf, southern California, by nitrogen and sulphur isotope ratios. *Marine Environmental Research*. 3: 215-224

Sweeney RE, Kalil EK, Kaplan IR (1980). Characterisation of domestic and industrial sewage in southern California coastal sediments using nitrogen, carbon, sulphur and uranium tracers. *Marine Environmental Research*. 3: 225-243

Tewfik A, Rasmussen JB, McCann KS (2007). Simplification of seagrass food webs across a gradient of nutrient enrichment. *Canadian Journal of Fisheries and Aquatic Science*. 64: 956-967

Tucker T, Sheats N, Giblin AE, Hopkinson CS, Montoya JP (1999). Using stable isotopes to trace sewage-derived material through Boston Harbor and Massachusetts Bay. *Marine Environmental Research.* 48: 353-375

Tveite S (1984). 0-group cod investigations on the Norwegian Skagerrak coast. The propagation of cod Gadus morhua L. *Flodevigen Rapportser*. *Series.* 1: 582-590

Udy JW, Bunn SE (2001). Elevated ^{15}N values in aquatic plants from cleared catchments: why? *Marine and Freshwater Research*. 52: 347-351

Udy J, Dennison W (1997). Physiological responses of seagrasses used to identify anthropogenic nutrient inputs. *Marine Freshwater Research*. 48: 601-604

Van Dover CL, Grassie JF, Fry B, Garritt RH, Starczak VR (1992). Stable isotope evidence for entry of sewage-derived organic material into a deep-sea food web. *Nature* 360: 153-155

Tveite S (1984) 0-Group cod investigations on the Norwegian Skagerrak coast. p. 581-590 *In*: E Dahl (ed) The propagation of cod, *Gadus morhua* L. Institute of Marine Research, Bergen, Norway.

Umezawa Y, Miyajima T, Yamamuro M, Kayanne H, Koike I (2002). Fine scale mapping of land-derived nitrogen in coral reefs by $\delta^{15}N$ in macroalgae. *Limnology and Oceanography*. 47: 1405-1416

Van Dover CL, Grassie JF, Fry B, Garritt RH, Starczak VR (1992). Stable isotope evidence for entry of sewage-derived organic material into a deep-sea food web. *Nature* 360: 153-155

Vander Zanden MJ, Vadeboncoeur Y, Diebel MW, Jeppensen E (2005) Primary consumer stable nitrogen isotopes as indicators of nutrient source. *Environmental Science & Technology*. 39: 7509-7515

Vitousek PM, Mooney HA, Lubchenco J & Melillo JM (1997) Human domination of earth's ecosystems. *Science*. 277: 494-499

Waldron S, Tatner P, Jack I, Arnott C (2001). The impact of sewage discharge in a marine embayment: a stable isotope reconnaissance. *Estuarine, Coastal and Shelf Science*. 52: 111-115

Walmsley, R.D. 2000. Perspectives on eutrophication of surface waters: Policy/research needs in South Africa. WRC Report No. KV 129/00. Water Research Commission, Pretoria, South Africa.

Wakelin SA, Colloff MJ, Kookana RS (2008) Effect of wastewater treatment plant effluent on microbial function and community structure in the sediment of a freshwater stream with variable seasonal flow. *Applied and Environmental Microbiology*. 74: 2659-2668

Wainwright SC, Fuller CM, Michener RH, Richards RA (1996). Spatial variation of trophic position and growth rate of juvenile striped bass (*Morone saxatilis*) in the Delaware River. *Canadian Journal of Fisheries and Aquatic Science*. 53: 685-692

Wainright SC, Haney JC, Kerr C, Golovkin AN, Flint MV (1998).Utilization of nitrogen derived from seabird guano by terrestrial and marine plants at St. Paul, Pribilof Islands, Bering Sea, Alaska. *Marine Biology*. 131: 63-71

Wallentinus I (1984). Comparisons of nutrient uptake rates for Baltic macroalgae with different thallus morphologies. *Marine Biology*. 80: 215-225

Wayland M, Hobson KA (2001). Stable carbon, nitrogen, and sulfur isotope ratios in riparian food webs on rivers receiving sewage and pulp-mill effluents. *Canadian Journal of Zoology*. 79: 5-15

Widory D, Kloppmann W, Chery L, Bonnin J, Rochdi H, Guinamant J-L (2004). Nitrate in groundwater: an isotopic multi-tracer approach. *Journal of Contaminant Hydrology* 72: 165-188

Yamamuro M, Kayanne H, Yamano H (2003). $\delta^{15}N$ of seagrass leaves for monitoring anthropogenic nutrient increases in coral reef ecosystems. *Marine Pollution Bulletin*. 46: 452-458

Zieman J, Macko S, Mills A (1984). Role of seagrass and mangroves in estuarine food webs: temporal and spatial changes in stable isotope composition and amino acid content during decomposition. *Bulletin of Marine Science* 35: 380-392

Zimmerman AR, Canuel EA (2000) A geochemical record of eutrophication and anoxia in Chesapeake Bay sediments: Anthropogenic influence on organic matter composition. *Marine Chemistry*. 69: 117-137

Zink K-G, Furtado ALS, Casper P, Schwark L (2004). Organic matter composition in the sediment of three Brazilian coastal lagoons – district of Macaé, Rio de Janeiro (Brazil). *Anais da Academia Brasileira de Ciências*. 76: 29-47