# THE NUTRIENTS DYNAMICS OF AN AUSTRALIAN RIVER SYSTEM AS MEASURED USING STABLE ISOTOPES.

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for the degree of Doctor of Philosophy.

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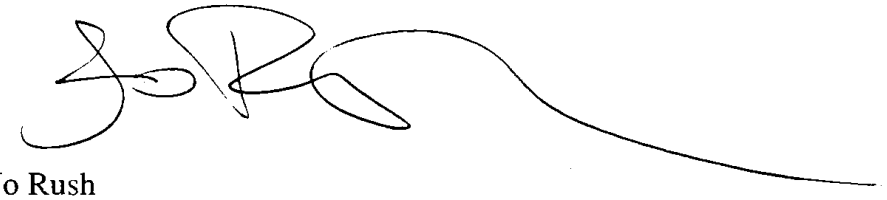

Jo Rush

*For Dad.*

# ABSTRACT

In Australia about 80% of the human population lives within 50 km of the coast and the trend to increasing development in coastal areas continues unabated. This places great stresses on coastal aquatic ecosystems from coastal rivers through estuaries to coastal marine waters. One of the consequences of this increasing urbanisation is eutrophication of coastal waters, the organic enrichment of aquatic ecosystems caused principally by increasing inputs of nutrients. This is not restricted to Australia alone but is a worldwide phenomenon leading to a range of problems in water bodies such as phytoplankton blooms, anoxia of sediments and bottom waters, declines in seagrasses and excessive growth of macroalgae in shallow estuaries.

In order to develop management strategies for coastal rivers and estuaries, diagnostic tools are needed to be able to identify the principal sources of nutrients to these systems and their relative impact on the food web starting with primary producers. The studies reported in this thesis use stable isotope analysis to identify the major sources of nitrogen to a coastal river system, the Hawkesbury-Nepean in central New South Wales, Australia and how the N from those sources is incorporated into algal biomass. An algal bioindicator is developed to overcome some of the problems associated with analysis of wild organisms and methods are proposed for correcting stable isotope incorporation into biomass for environmental effects.

In the Hawkesbury-Nepean River, eutrophication, resulting from high loads of nitrogen and phosphorus, has been a persistent problem. Shifts in management practices have led to a substantial reduction in the P load of the river, but many of the problems associated with eutrophication remain. The focus has now shifted to the control of N sources. In order to adequately manage the issue of nitrogen-enriched water bodies, it is essential to be able to identify the main sources contributing to the excess load of N. Stable isotope ratios such as $\delta^{15}N$ may be useful in these types of "tracer" experiments. However, there are a number of sources of error inherent in this type of study. Trophic level, genetic differences, N storage strategies, biochemical differences and response to environmental conditions, as well as N source, can all affect the final $\delta^{15}N$ of an organism. In some cases, extraneous sources of $\delta^{15}N$ variation may be large enough to mask changes in $\delta^{15}N$ due to N source changes.

In order to successfully trace the main sources of N in an ecosystem, it is also essential to know the $\delta^{15}N$ of each source. In the Hawkesbury-Nepean River, 3 major sources of N have been identified: Effluent from sewage treatment plants (STPs), run-off from agricultural land in the catchment, and N stored and recycled from the sediments. Each of these sources was characterised in terms of their mean $\delta^{15}N$ value in a series of experiments. Effluent from STPs was found to have a very high mean $\delta^{15}N$ of 15.6‰ and agricultural run-off a low mean $\delta^{15}N$ of 4.1‰. Sediment mean $\delta^{15}N$ lay between these two values, at 10.0‰. Each source had a distinct range of $\delta^{15}N$ values, allowing it to be reliably distinguished from the others by way of $\delta^{15}N$ values. These values were used in the interpretation of $\delta^{15}N$ data in a green alga, *Microdictyon umbilicatum*, grown in the river during a number of field trials over a period of 3 years.

In this thesis, the green alga *Microdictyon umbilicatum* was developed as a bioindicator to take $\delta^{15}N$ samples from the Hawkesbury-Nepean River. This eliminated many of the potential sources of variation in $\delta^{15}N$ values. Nitrogen storage, nutrient concentration, light levels, temperature and salinity were identified as factors potentially altering the level of fractionation between *M. umbilicatum* and its N source. These factors were investigated in a series of laboratory experiments. *Microdictyon umbilicatum* was found to turn over all N within 14 days in both laboratory and field conditions, thus eliminating the problem of stored N diluting the new N pool $\delta^{15}N$. Temperature and salinity, within the ranges encountered in the river, were found to have no effect on the final $\delta^{15}N$ of *M. umbilicatum*. Nitrate concentrations above 5mg.mL$^{-1}$ were found to increase fractionation in *M. umbilicatum* from -1‰ (seen under normal environmental conditions) to a mean of -2.7‰. Low light levels also increased fractionation in *M. umbilicatum*, to a mean of –3.0‰. Field data collected from sites with high nitrate concentrations or low light levels were therefore adjusted as appropriate to allow for increased fractionation between source N and the plant.

STPs were found to heavily influence the $\delta^{15}N$ of indicator plants grown in the river for up to 30km downstream of outfalls. Rainfall, which would be expected to greatly increase the contribution of agricultural run-off to the N load of the river, did not affect the general pattern of very high $\delta^{15}N$ values near STP outfalls and downstream. The contribution of STPs to the eutrophication of the Hawkesbury River is estimated in this thesis to be considerable and consistently the greatest contributor to the bioavailable N load of the river system.

This thesis has developed the first insight into the relative contribution of different N sources to an Australian estuary using a stable isotope technique. It has developed a protocol based on a laboratory-cultured indicator species for collecting $\delta^{15}N$ data from aquatic ecosystems. It also highlights the importance of controlling or estimating the effect of extraneous factors so as to reduce variability and increase the precision, and reliability of $\delta^{15}N$ data. Most importantly, it develops a solid theoretical basis for the use of $\delta^{15}N$ values in determining the source of excess N contributing to eutrophication. The $\delta^{15}N$ bioindicator developed in this thesis will have wide application in determining the source of nutrient pollution contributing to a decline in water quality in many aquatic ecosystems.

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# CHAPTER 1: INTRODUCTION.

## 1.1 General introduction

Water quality and aquatic pollutants have become major issues world-wide. The United Nations 1999 "Global Environmental Outlook" report lists water quality and marine pollution as one of four key global environmental problems. The report highlights pollution, in particular excess nutrients, as a major problem in the Baltic, North, Black, Mediterranean, Caspian and Adriatic seas and on the west coasts of Sweden and Denmark (UNEP, 1999). As the population centres of the world become increasingly urbanised, nutrient pollution associated with the raising of food crops, livestock and the waste products in sewage effluents becomes an acute problem. Population centres tend to be associated with water bodies, with over half the world's population, or approximately 3.4 billion people, living within 60km of the ocean (Prescott, 1998). Such dense populations in coastal areas places enormous stress on aquatic ecosystems. Less than 50% of the population in Europe, America and Asia are serviced by any form of wastewater treatment facility, with this dropping to less than 2% of the population in many developing countries (WRI, 1999). In areas with no wastewater treatment, the vast bulk of sewage goes untreated into the nearest water body. It is therefore not surprising that anthropogenic nutrient pollution is now almost ubiquitous in rivers, estuaries and marine water bodies world-wide, with 70–80% of all marine pollution estimated to be terrestrial in origin (Claussen, 1997; UNEP, 1999).

In undisturbed water bodies, inorganic nutrients in forms available to aquatic organisms such as nitrogen and phosphorus are generally in short supply. Aquatic organisms are well adapted to this situation, and many have developed sophisticated mechanisms for the uptake and recycling of nutrients. The enrichment of inorganic nutrients, usually N and P, in the environment leads to eutrophication (BALLERINA, 1999, National Research Council, 2000).

Eutrophication is defined as the enrichment of aquatic ecosystems by organic carbon, frequently algae associated with an excess of bioavailable nutrients, which reduces the oxygen dissolved within a body of water, producing an environment which can can severely disturb aquatic ecosystems, both on an individual organism and community level (Chesapeake Bay Critical Area Commission, 2001; Davis and Koop, in press). Anoxia, toxic algal blooms, disruption of sediment processes and the simplification of community structure are among the more common results of eutrophication (BALLERINA, 1999; Davis and Koop, in press).

*Nutrient sources*

Excess nutrients may originate from a variety of sources. Wastewater from industries, sewage effluent, storm water, agricultural run-off, shipping wastes and aerial pollution can all contribute to the load of nutrients entering coastal aquatic ecosystems (Boynton, 1997; Environment Australia, 1997a; UNEP, 1999). Many of these sources add large amounts of nitrogen and phosphorus to the system. For example, acid rain alone contributes 5-10 kg of $N^{-1}/acre^{-1}/year^{-1}$ in the US Chesapeake Bay catchment (Boynton, 1997). In the Hawkesbury-Nepean River, anthropogenic inputs from the city of Sydney add approximately 600 tonnes of nitrogen and 50 tonnes of phosphorus to the river and estuary per year (NSW EPA, 1994).

*Results of eutrophication*

Eutrophication can add to the degradation of water quality and aquatic ecosystems in many ways. In rivers, estuaries and coastal marine waters, eutrophication can contribute to mass blooms of algae and dinoflagellates, which greatly reduce water quality and biodiversity (Harris, 1986; NSW EPA, 1997; Koop *et al.*, 1998). Blooms block sunlight, absorb oxygen

from the water column and release chemicals which are toxic to fish, shellfish and mammals. Neurotoxins, hepatotoxins and lipopolysaccharides have all been isolated from algae and dinoflagellate species, and have caused the death of mammals, birds, fish and amphibians in affected waterways (NRA, 1990; Hallegraeff, 1993; Lassus *et al.*, 1995; Davis, 1997; MBARI, 1998). In May 1998, hundreds of sea-lions were killed on Campbell and Auckland Islands, off New Zealand, by toxic dinoflagellate blooms (NCWAR, 1998). There are no confirmed reports of human death directly attributable to ingesting toxic blue-green algae, but there are numerous reports of illness caused by contact with blooms (e.g. Hallegraeff, 1993; MBARI, 1998; UNEP, 1999). These include skin irritations and allergic reactions in swimmers (e.g. Lassus *et al.*, 1995; NCWAR, 1998), hepatoenteritis in individuals who have consumed water infected with blue-green algae (NRA, 1990; Lassus *et al.*, 1995) and gastrointestinal illness in individuals who have swallowed small quantities of algae during water sports (Hanisak, 1983; NRA, 1990; Davis, 1997; NSW EPA, 1997). Toxic algal blooms severely reduce water quality and make affected areas unsuitable for recreation, drawing of drinking water or stock access. Algal blooms in Australian freshwaters have been estimated to cost the community between $180 and 240 million per year (Atech, 2000)

In 1998 alone, toxic blooms were reported in Australia, the USA, Finland, Sweden, Norway, Chile, New Zealand and the UK (MBARI, 1998; UNEP, 1999). In November 1991, more than 1000 km of the Barwon and Darling Rivers in NSW were affected by a bloom of toxic cyanobacteria (Sarne, 1999). As rivers, estuaries and marine systems become increasingly eutrophic, and as flows are reduced by damming and water extraction, toxic blooms have become increasingly common, both in Australia (Davis, 1997; NSW EPA, 1997) and world-wide (Hallegraeff, 1993; Lassus *et al.*, 1995).

All aquatic plants grow and die at accelerated rates in nutrient-rich waters (UNEP, 1999). This results in increased plant biomass, which can alter plant communities and create boating or recreational hazards as well as being aesthetically displeasing. Exotic aquatic macrophytes are often not subject to the same constraints from excess nutrient levels as native plants, and therefore easily displace endemic macrophytes in eutrophic waters (Hanisak, 1983; NSW EPA, 1994; Koop, 1997; Davis and Koop, in press). In many areas of the Hawkesbury-Nepean River, the exotic aquarium plant, *Egeria densa* has replaced native plants such as *Vallisneria sp.*, blocking waterways, obstructing navigation and increasing sedimentation (Koop, 1997). Excess macrophyte growth also reduces light penetration and provides a physical barrier to fish migration, fishery activities or water extraction.

Eutrophication can also cause hypoxia of the water column and sediments. Increased plant or algal biomass, stimulated by nutrient inputs, creates an excess of dead material. This material subsequently decomposes, using the majority of oxygen in the water column (SEAC, 1996). During the day, live plant material may to some extent counter this tendency toward anoxia due to the photosynthetic release of oxygen into the water column (Rogers and Gallon, 1988). However, respiration reactions in the dark consume oxygen and may in fact exacerbate hypoxia and resulting effects on organisms (Rogers and Gallon, 1988; Hallegraeff, 1993). Some nitrogen compounds released into the river react with oxygen in the water column, reducing the amount of free oxygen available to organisms (NSW EPA, 1994). Reduced oxygen levels favour hardy introduced fish species such as carp over native species and in extreme cases can result in blanket fish kills (NRA, 1990; Davis, 1997; Koop, 1997; Davis and Koop, in press).

Excess nutrients in aquatic systems can also disturb the delicate balance between grazing zooplankton and microalgae. This leads to the disruption of food chains and consequently has repercussions for fisheries and water quality (Scholten, 1997). Sediment communities can also be changed by an input of nutrients, with addition of nitrates changing macroinvertebrate populations to the extent that community structure is proposed as an indicator of nitrate pollution (H. Surtikanti, University of Technology, Sydney, pers. comm.).

The effects of excess nutrients are particularly evident in temperate seagrass communities. Vast declines in the distribution and density of native seagrass beds have been recorded at many sites subject to nutrient enrichment (Larkum, 1976; Stevenson *et al.*, 1993; Horrocks *et al.*, 1995; reviewed in Koop, 1997). As seagrass beds provide an important nursery area for many commercially important marine species, their absence can be detrimental to fisheries.

Eutrophication also impacts severely on tropical marine ecosystems. The degradation of tropical coral reefs is accentuated by excess nutrients in these naturally oligotrophic ecosystems (Hatcher *et al.*, 1989; Dubinski *et al.*, 1990; Hoegh-Guldberg *et al.*, 1996; Takabayashi, 1996; Hoegh-Guldberg, 1999). Eutrophication can also enhance the survival rates of planktotrophic marine larvae, contributing to mass population fluctuations of species such as *Acanthaster* (the reef-destroying Crown-of-Thorns starfish). Eutrophication has been speculated (although not proven) to be the major cause of Crown-of-Thorns starfish outbreaks and their associated effects on coral reef communities (Birkeland, 1982; Hatcher *et al.*, 1989; Moran and Davies, 1989).

*The importance of aquatic ecosystems*

Aquatic environments are extremely important on a global scale – biologically, economically, socially and as a source of essential food for much of the developing world. It is estimated that 950 million people world-wide obtain their primary source of protein from seafood (WRI, 1999). Australia's aquatic resources contribute greatly to the nation's economy. In 1994, marine tourism and recreation were worth $15 billion, recreational fishing $3 billion and commercial fishing and aquaculture $1.6 billion, overtaking the financial contributions of traditional export industries like coal mining and oil extraction (SEAC, 1996). All of these activities rely upon the health of our aquatic ecosystems, either directly (fishing) or indirectly (tourism and recreation). Ninety percent of Australia's population live within 50km of the coast, and water bodies play a strong cultural role for many people (Australian Bureau of Statistics, 1999). Awareness of aquatic pollution problems such as eutrophication is growing rapidly throughout the community. A 1992 survey in Australia showed that 75% of people were concerned about the environment (Environment Australia, 1997b). Research into the social and cultural value of the aquatic environment to Australians is scarce, however, the emergence of user groups and numerous environmental organisations indicates strong community concern about aquatic ecosystems.

*Nitrogen compounds and aquatic ecosystems*

Nitrogenous compounds are essential and often limiting nutrients in marine ecosystems, and also have a major influence on the chemistry of the atmosphere. Unfortunately, as well as being one of the most important element cycles, nitrogen cycling is also one of the most vulnerable to human influences. For example, industrial fixation of N into fertiliser is now approximately equal in magnitude to natural biological N fixation (Heaton, 1986). Together

with effluents from sewage and industry, this fertiliser may pollute marine ecosystems with excess N compounds.

Excess aquatic nutrients are generally in the form of nitrogen (largely as ammonium and nitrate) and phosphorus (organophosphates and particle-associated phosphates). The bulk of these compounds come from sewage and run-off from land used for various types of agriculture (NSW EPA, 1993 & 1994; SEAC, 1996). Identifying the sources of excess nutrients and their relative contribution to eutrophication in each ecosystem is vital to formulating effective plans to manage this issue. In Australian waterways, N seems to be of major importance in limiting phytoplankton production, with recent data (Oliver et al., 2000) suggesting that N is the limiting nutrient in the Darling River and in other Australian rivers as well (Robertson, 1999; Davis and Koop, 2001). The source of nitrogen compounds found in the environment can be identified using stable isotope analysis (natural variations in the ratio of $^{15}N$:$^{14}N$), and it is this method which is the focus of this thesis.

## 1.2 The use of stable isotopes of nitrogen in ecological studies

### *1.2.1 Nitrogen stable isotopes*

Elements can exist as both stable and unstable (radioactive) isotopes. Most elements of biological significance have at least two stable isotopes, generally a common lighter isotope and a rare heavier one. In the case of nitrogen, $^{14}N$ makes up 99.63% of total terrestrial nitrogen, with $^{15}N$ comprising the remaining 0.37% (Ehleringer and Rundel, 1989). The average natural abundance of $^{15}N$ in air remains constant at 0.366% (Nier, 1950; Sweeney *et al.*, 1978) and is used as the standard for nitrogen analyses.

The ratio of $^{15}N$: $^{14}N$ in a sample is therefore expressed as:

$$\delta^{15}N\ (‰) = [(^{15}N/^{14}N_{sample}/^{15}N/^{14}N_{air}) - 1] \times 10^3 \qquad (1)$$

Variation in the ratio between the isotopes depends on the thermodynamic equilibrium and any kinetic processes affecting the abundance of each isotope. A change in the ratio of $^{15}N$: $^{14}N$ between compounds is referred to as fractionation. Fractionation is the result of slight variations in the physical and chemical properties of the two isotopes that leads to slight differences in how these isotopes behave (Ehleringer and Rundel, 1989). Enzymatic processes often discriminate for or against an isotopic species, producing a product that is either enriched or depleted in one isotope (Mariotti *et al.*, 1981; Macko *et al.*, 1986; Blackburn and Knowles, 1993). The factors influencing isotope fractionation are further discussed in Chapter 2.

Generally, animal tissues are enriched in $^{15}N$ compared to plants and this enrichment increases at an average of 3.3 ‰ per trophic level (Wada *et al.*, 1987). This is due to catabolic

pathways which favour the elimination of the lighter isotope (Epstep and Vigg, 1985; Yoshioka and Wada, 1994; Peterson *et al.*, 1995; Hoefs, 1997). Effectively, this means that in general, the greater the number of biological processes that a substance undergoes, the higher its $\delta^{15}N$ value. $\delta^{15}N$ values in nature range from –20‰ to +20‰ (Figure 1.1).

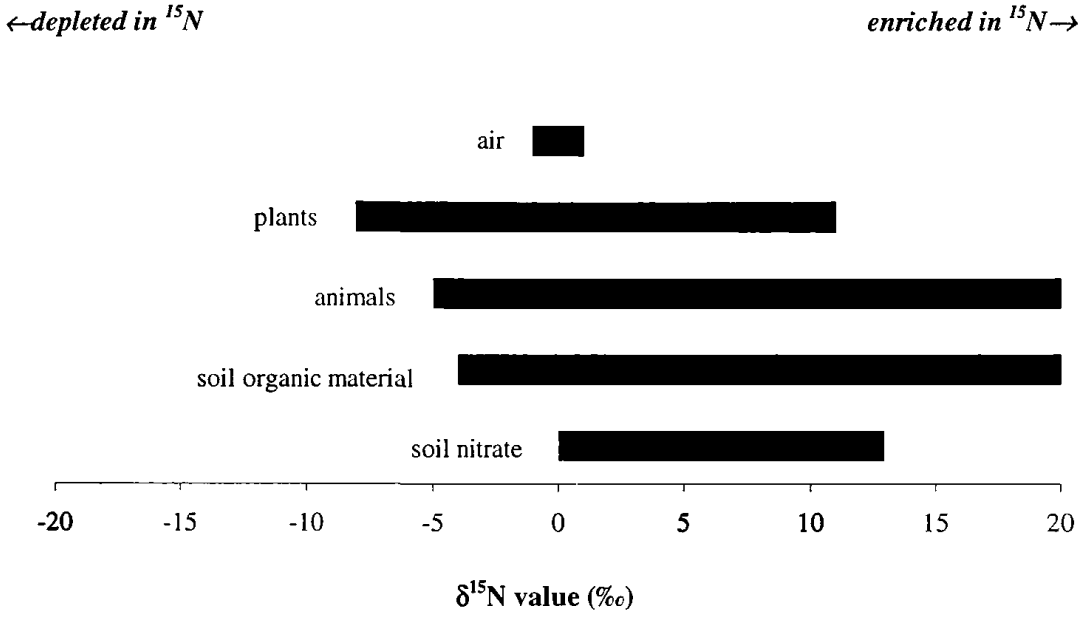



**Figure 1.1**: Ranges of $\delta^{15}N$ values of various substances (adapted from Ehleringer and Rundel, 1989).

Stable isotopes of nitrogen are a useful tool in ecological studies because of the discrimination against one isotope by various biological processes. Food webs can be elucidated using $\delta^{15}N$ data, with organisms from higher trophic levels being enriched in $^{15}N$ compared to organisms from lower trophic levels (Hoch *et al.*, 1992; Yoshioka and Wada, 1994). Nutrient inputs high in $^{15}N$ can be traced through an ecosystem due to the corresponding high $\delta^{15}N$ values in organisms that have assimilated or consumed them (Blackburn and Knowles, 1993; Lindau *et al.*, 1996). Levels of nitrogen fixation from the atmosphere can also be estimated from $\delta^{15}N$

values (Mariotti *et al.*, 1981; Shearer and Kohl, 1989). However, there are many limitations to the interpretation of these kind of data (for review, see Handley and Scrimgeour, 1997). Some of the advantages and disadvantages of using stable isotopes in ecological studies are discussed in the next Section and in detail in Chapter 2.

### *1.2.2 Stable isotopes in ecological studies*

Stable isotope pairs, most commonly $^{15}N$:$^{14}N$, $^{18}O$:$^{16}O$ and $^{13}C$:$^{12}C$, have been used extensively in ecological studies for over 25 years. Generally, stable isotopes can be used to define food web patterns, or to trace the fate of a nutrient input through an ecosystem. Nitrogen isotope data can provide a powerful tool for obtaining insights into these types of questions when correctly interpreted. However, caution is needed when applying these data to ecological processes, due to the large number of potentially confounding factors that are inherent in these types of studies. From the first widely published ecological study utilising $^{15}N$ as a "tracer" of fertiliser-derived nitrate (Kohl *et al.*, 1971), doubts have been raised as to the validity of some interpretations of $\delta^{15}N$ data (Hauck *et al.*, 1972). Many of the criticisms of this work centred on the high probability of fractionation between N source and sink, and the difficulties in using a rapidly changing signal as a "tracer".

Numerous studies have been done using unverified interpretations of $\delta^{15}N$ data. Many researchers have pointed out the multiplicity of processes that can lead to similar $\delta^{15}N$ values, and warned of the need for careful manipulative experimentation and hypothesis testing to isolate the process of interest (e.g. Mariotti *et al*, 1981; Hoch *et al.*, 1992; Blackburn and Knowles, 1993; Handley and Scrimgeour, 1997; Handley *et al.*, 1998). However, these recommendations do not seem to have been widely adopted by ecological researchers. Many studies make the false assumption that $\delta^{15}N$ is a reliable tracer of N from soil to leaves, and

interpret leaf values as reflecting the source (e.g. Heaton, 1987; O'Donahue and Dennison, 1997). Other studies have over-interpreted $\delta^{15}N$ results in terms of food webs (e.g. Epstep and Vigg, 1985; McClelland, 1997; Fry *et al.*, 1999) and there are numerous examples of studies where $\delta^{15}N$ has been used as a "tracer" with little recognition of the possible confounding factors (e.g. Edwards, 1973; Mulholland and Olsen, 1992; Lowe, 1996). These factors are discussed in detail in Chapter 2.

This thesis explores the use of stable isotope data and incorporates the recommendations of others working in this area to refine the techniques used to collect and interpret $\delta^{15}N$ data in an aquatic ecosystem. The principle focus of this thesis was the complex flows of nitrogen within an Australian estuary. The Hawkesbury-Nepean River was selected for this study, as it was ideally situated because of the distinct nature of the sources of isotopes flowing through the system. For this reason, the Hawkesbury-Nepean also served as an ideal model for developing a technique based on stable isotopes for tracing nutrient sources within an estuary.

The question of nutrient sources is crucial in managing the Hawkesbury-Nepean River. Parts of the river system have been plagued by chronic algal blooms. In other areas, introduced aquatic weeds have all but choked the waterway, presumably due to the increased flow of nutrients to the watershed. Management of the river is largely aimed at reducing the effects of eutrophication, but it is important to identify the main factors contributing to these effects. In order to develop relevant and cost-effective nutrient management strategies for the river, it is necessary to quantify the relative contributions of sewage treatment plants, stormwater and agricultural run-off before committing to expensive long-term measures. Data collected during the course of this project is therefore immediately relevant to a specific management problem, as well as the developed technique itself having a wide range of applications.

## 1.3 The Hawkesbury-Nepean River system

### *1.3.1 General description*

The Hawkesbury–Nepean River system, located near Sydney, NSW, drains a catchment of approximately 22,000 $km^2$. The Nepean River rises near the town of Robertson in the Illawarra Range and flows northward for 113 km. At the confluence with the Grose River it becomes known as the Hawkesbury River, which flows north and then east a further 143 km to Broken Bay (Figure 1.3). This large river system has been an integral part of the rapid development of the Sydney metropolis since European settlement. It is highly regarded by the community for a range of potential uses or environmental values which include habitat for aquatic flora and fauna, recreational and agricultural uses, stocks of edible fish and shellfish and as a supply of potable and non-potable water.

Because of the river's close proximity to a large and growing city, the quality of its water has been, and continues to be, compromised by the addition of pollutants from urban, rural and industrial runoff and sewage effluent. Compounding this problem has been the reduction in diluting river flows resulting from the damming of the upper Nepean River and four of its major tributaries. These dams supply Sydney and surrounding areas with drinking water. The addition of pollutants and the reduction in river flows has put the Hawkesbury-Nepean River system under stress.

While more than 50% of the 22,000 $km^2$ catchment area is forested (NSW EPA, 1994; HNCMT, 1999; Streamwatch, 1999), agricultural activity (alone worth over $1 billion per year; HNCMT, 1999) and urban and industrial development is extensive.

The Colo, Macdonald, Mangrove and Blue Mountains sub-catchments are largely rugged natural bush land protected by national parks, nature reserves, protected water catchments and proposed wilderness areas. The Cumberland Plain, largely contained within the South Creek and Middle Nepean sub-catchments, has been substantially cleared of native vegetation and supports major agricultural industries. The area also supplies significant sand and gravel resources and is absorbing the majority of Sydney's urban expansion. The South and Western areas of the catchment contain a mix of agriculture, forestry, mining and quarrying enterprises as well as significant natural bush land areas (Figure 1.2).

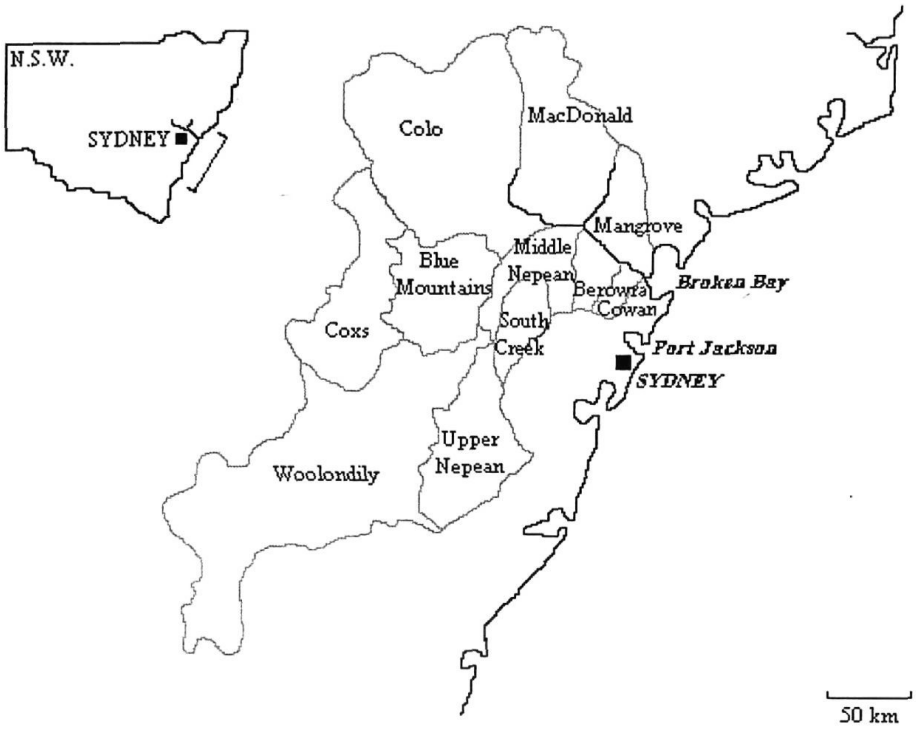



**Figure 1.2**: Map of the Hawkesbury-Nepean catchment area (adapted from HNCMT, 1999).

The Hawkesbury-Nepean River's flow is heavily regulated by dams and weirs. Five of the river's major tributaries are dammed, the largest of these being Warragamba Dam which supplies water to approximately 95% of the greater Sydney region (Deen and Maheswaran, 1998). Damming and extraction of water reduces the rivers flow and current velocity. Flow is a key parameter affecting water quality and its reduction tends to exacerbate other factors contributing to the degradation of waterways (NSW EPA, 1994).

Turbidity is also a problem at some sites in the Hawksbury-Nepean River. Reduced clarity can affect the aesthetic suitability of the water for swimming or recreation, the ability of organisms to find their prey and the degree of light penetration, which in turn affects plant growth (NSW EPA, 1994; Douglas *et al.*, 1997). Turbidity (measured as Secchi depth) data

varies greatly between sites and at different times in the Hawkesbury-Nepean River. Generally however, turbidity is highest within and near the confluence of tributaries with the bulk of their flow consisting of sewage treatment plant effluent (South, Rickabys and Cattai Creeks; NSW EPA, 1994; Figure 1.3).

Both diffuse and point sources contribute large amounts of nutrients to the Hawkesbury-Nepean River system. The river and catchment, particularly the downstream areas, are affected by storm water run-off from urban, industrial and agricultural lands (diffuse sources) and direct discharges of wastewater (point sources). The latter are primarily due to effluent from sewage treatment plants (STPs) that are situated at various points along the river. The river currently receives the treated effluent from over 800, 000 residents, and the population of the area is projected to rise to over one million by the year 2021 (Deen and Maheswaran, 1998; Streamwatch, 1999). Major STPs discharge into the river at South Creek, Eastern Creek and Cattai Creek in the South Creek catchment (Figure 1.3; NSW EPA, 1993 & 1994).

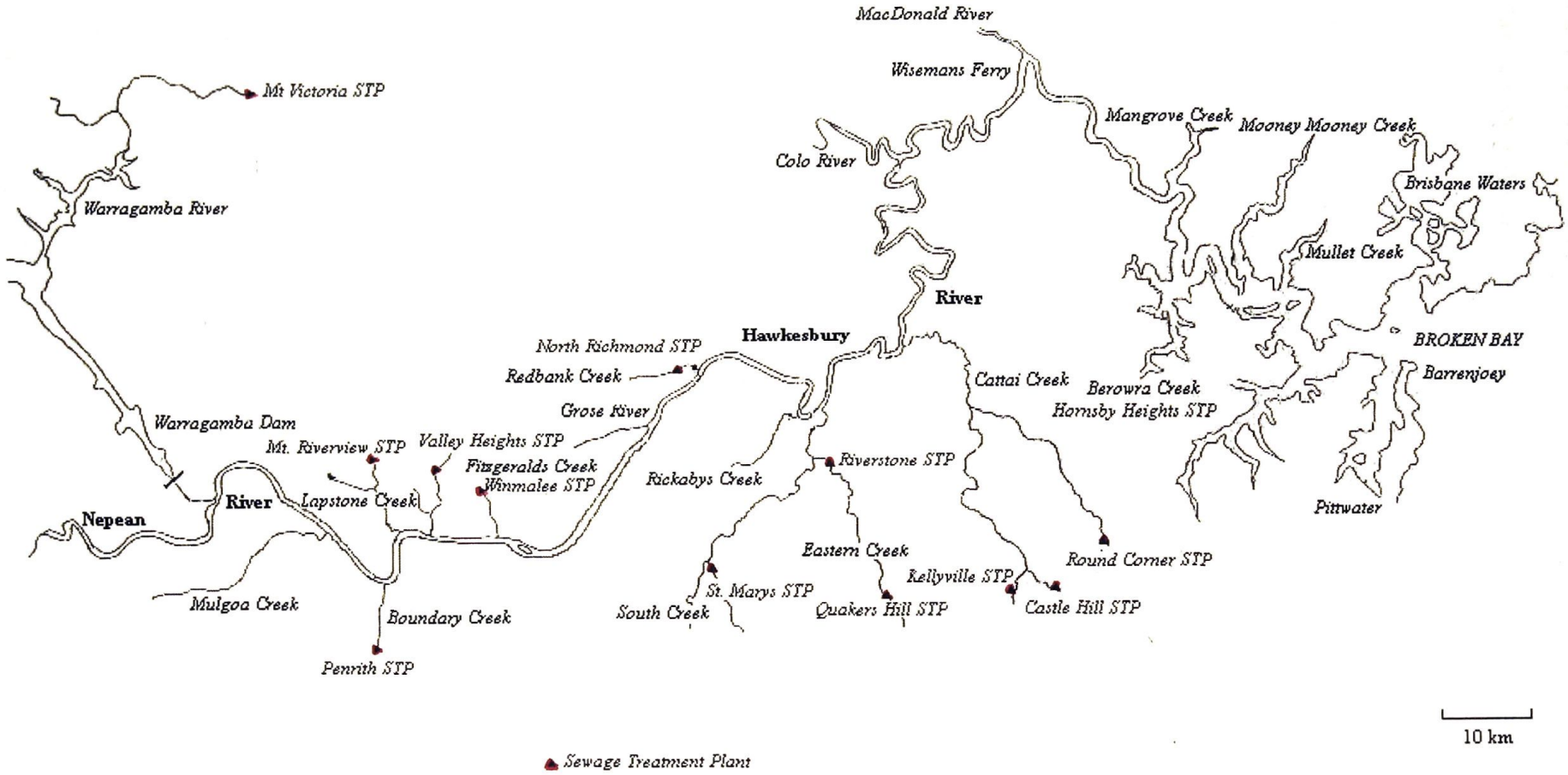



**Figure 1.3**: Map of the Hawkesbury-Nepean River catchment showing major tributaries and sewage treatment plants in the catchment (adapted from NSW EPA, 1994).

### *1.3.2 Water quality in the Hawkesbury-Nepean River*

Eutrophication was first identified as a major problem in the Hawkesbury-Nepean River in the late 1970's and has become an increasing problem in recent years. Eutrophication contributes to the appearance of nuisance levels of free-floating and attached macrophytes and frequent phytoplankton blooms, some of which are toxic. Oxygen depletion of the water column is also a problem at some sites (Koop, 1997; Davis and Koop, in press).

Effluent from sewage treatment plants (STPs) has been identified as the cause of eutrophication and the subsequent reduction in water quality (SPCC, 1983). In response to findings by the State Pollution Control Commission (SPCC) in a study conducted from 1979-1981, a management strategy was implemented to reduce the detrimental effects of STP effluent on water quality in the Hawkesbury-Nepean River.

Strategies recommended by the SPCC (1985) at the STPs included:

1. Aeration of effluent. This has two consequences:

   a) reducing the oxygen demand of the effluent once it is discharged to the river by providing oxygen for reactions in the plant.

   b) reducing ammonium levels in the effluent by supplying oxygen for the nitrification reaction (carried out by specialist bacteria), which converts toxic ammonia to nitrate.
2. Phosphorus removal via chemical precipitation or biological processes to reduce the amount of phosphorus contributing to eutrophication.
3. Denitrification to reduce the amount of nitrogen in the effluent contributing to the eutrophication of the river.

These recommendations have been largely implemented, with the majority of STP effluent now being treated using all 3 strategies (NSW EPA, 1993 & 1994; HNCMT, 1999). Phosphorus removal has been particularly successful, with phosphorus loads in the Hawkesbury-Nepean River decreasing notably since phosphorus removal measures were introduced. However, although ammonia levels have been reduced, the overall nitrogen load remains unchanged from 1981 and still greatly exceeds water quality criteria in the majority of sites that are monitored (NSW EPA, 1994).

Many of the problems associated with eutrophication also remain. In particular, algal blooms are still a significant problem in the Hawkesbury-Nepean River. In the period from 1996 to 1999, approximately 75% of weekly algal density measurements in the Hawkesbury-Nepean River reported medium or high densities of microalgae at one or more sites (NSW EPA, 1999). This was more frequent than in any other monitored river in the state. One bloom of blue-green algae at Sackville persisted from December 1996 to the end of March 1997 (HNCMT, 1999).

Blooms of N-fixing cyanobacteria, which were thought to be largely limited by the availability of phosphorus, remain a problem in the Hawkesbury-Nepean River. Blooms of non-fixing species, which are reliant on N from the water column, are also becoming increasingly common (Harris, 1996; Koop, 1997).

### *1.3.3 The contribution of phosphorus and nitrogen to eutrophication of the Hawkesbury-Nepean River*

In undisturbed fresh water, phosphorus is generally deficient and therefore limits plant growth. Excess phosphorus enters waterways in run-off from agricultural land and in discharges from point sources. Because of its capacity to be adsorbed by reactive surfaces, much of the phosphorus in run-off tends to be associated with particles, and is therefore largely unavailable for use by plants. Phosphorus from run-off is therefore unlikely to be the major contributor to excessive plant growth in the Hawkesbury-Nepean River. In contrast, approximately 70% of the phosphorus in discharges from STPs is in the form of soluble orthophosphate, which is readily available to plants (NSW EPA, 1994). To control plant growth, criteria for total phosphorus levels have been set (Table 1.1).

**Table 1.1**: Recommended levels of total phosphorus ($\mu g.L^{-1}$) to control eutrophication and the associated reduction in water quality (ANZECC and ARMCANZ, 2000).

| **Parameter** | **Australian Water Quality guidelines (ANZECC and ARMCANZ, 2000)** |
|---|---|
| ***Total phosphorus in fresh waters in SE Australian lowland rivers*** | 50 $\mu g.L^{-1}$ |
| ***Total phosphorus in marine waters*** | 25 $\mu g.L^{-1}$ (coastal waters)<br>20 $\mu g.L^{-1}$ (offshore waters) |

Although soluble phosphorus levels in many of the tributaries of the Hawkesbury-Nepean River (where STP effluent is released) are at levels above those recommended in the Australian water quality guidelines, dilution into the larger mainstream water body brings the level of soluble phosphorus to below the threshold level most of the time (NSW EPA, 1994). Despite this, excess macrophyte growth and algal blooms remain common in the mainstream of the Hawkesbury-Nepean River.

In undisturbed marine waters, including estuaries, nitrogen is often the limiting nutrient for plant growth, as phosphorus is generally available in excess in marine waters (AEC, 1987; NSW EPA, 1994). While N is required in small amounts by organisms, high levels of nitrogen compounds such as ammonia, nitrate and nitrite can be toxic to aquatic organisms, in particular fish. Nitrogen compounds enter the river from both diffuse run-off and STP effluent. Since the introduction of a nitrification step to the processing of sewage effluent, little ammonia enters the river from this source, the majority of nitrogen in effluent being in the form of nitrate (NSW EPA, 1994). Most of the nitrogen from run-off is also in the form of nitrate (Kreitler, 1979; Komor and Anderson, 1993; Hopkins *et al.*, 1998). However, nitrate can easily be reduced to ammonium under the right conditions in the river. To control excess plant growth, criteria for N compounds have been set in the Australian water quality guidelines (ANZECC and ARMCANZ, 2000; Table 1.2).

**Table 1.2**: Recommended levels of nitrogen compounds ($\mu g.L^{-1}$) to control eutrophication and the associated reduction in water quality (ANZECC and ARMCANZ, 2000).

| **Parameter (for SE Australia)** | **guidelines** |
|---|---|
| ***Total nitrogen in lowland rivers*** | 500 $\mu g.L^{-1}$ |
| ***Oxidised nitrogen in lowland rivers*** | 40 $\mu g.L^{-1}$ |
| ***Oxidised nitrogen in marine waters*** | 25 $\mu g.L^{-1}$ (higher than elsewhere in SE Australia because of periodic upwelling) |
| ***Ammonium in lowland rivers*** | 20 $\mu g.L^{-1}$ |
| ***Ammonium in marine waters*** | 20 $\mu g.L^{-1}$ (higher than elsewhere in SE Australia because of periodic upwelling) |

Since the introduction of nitrification processes in STPs, ammonium levels in the river are well below the proposed guideline levels. However, despite efforts to reduce the total amount of nitrogen entering the river from STP effluent, total nitrogen and nitrate levels in the Hawkesbury-Nepean River remain well above the recommended level at almost all sites (NSW EPA, 1994; HNCMT, 1999). Blue-green algal blooms are also frequent, despite some predictions that increasing the ratio of nitrogen: phosphorus would reduce the incidence of these blooms (Harris, 1994; NSW EPA, 1994).

Overall, total phosphorus loads in STP effluent have been reduced by an average of 61% from 1979 to 1999, although total nitrogen loads have remained approximately the same for this period (NSW EPA, 1994). This has effectively increased the nitrogen: phosphorus ratio, which is sometimes useful in controlling blue-green algal blooms by removing the competitive advantage of nitrogen-fixing cyanobacteria (D'Elia *et al.*, 1986; Harris, 1986). However, the complexities of algal population dynamics in the Hawkesbury-Nepean River

and the highly eutrophic waters have negated this effect and blooms of various types continue to be common (Harris, 1994).

A large body of evidence now exists to challenge the conventional wisdom that freshwater phytoplankton are limited by available phosphorus and marine phytoplankton are limited by nitrogen (Ryther and Dunstan, 1970; Grimm and Fisher, 1986; Harris, 1986 & 1994; Suttle and Harrison, 1988; Elser *et al*, 1990; Fisher *et al.*, 1992; Moss *et al.*, 1996; Koop, 1997). Nitrogen limitation in Australian rivers is common (Harris, 1994 & 1996; Douglas *et al.*, 1997; Grace *et al.*, 1997; Koop, 1997; Davis and Koop, in press), as is phosphorus limitation in marine waters (Fisher *et al.*, 1992; Koop *et al.*, 1998; Davis and Koop, in press).

Research (Hanisak, 1983; Nixon and Pilson, 1983; Harris, 1994 & 1996; Douglas *et al.*, 1997; Grace *et al.*, 1997; Koop, 1997; Oliver, 1997 Robertson, 1999; Oliver *et al.*, 2000; Davis and Koop, 2001), and the failure of reducing phosphorus levels alone to decrease the incidence of phytoplankton blooms in the Hawkesbury-Nepean River have highlighted the need for a simultaneous reduction of nitrogen levels to control the effects of eutrophication. This thesis has therefore concentrated on the contribution of nitrogen to the eutrophication of the Hawkesbury-Nepean River.

*Bioavailable nitrogen sources*

The relationship between nutrients and their ecological effects is a complex one. Increasingly, it has become evident that the traditional eutrophication model based on nutrient inputs alone must be replaced with one taking into account system-specific interactions between many complex ecosystem responses (Cloern, 2001). In particular, simply measuring the raw amounts of nutrients entering an ecosystem tells us little about the effects that these nutrients will have on the biota.

Nitrogen from sewage treatment plants is generally soluble and hence readily available for biological uptake, ie it is bioavailable (Gabric and Bell, 1993). In contrast, nutrients carried in run-off are though to be often tied up in less bioavailable forms. Analysis of the inorganic soluble forms of N in the water column appears to give an accurate estimate of bioavailable N, but does not allow the source of this ecologically important N to be identified. This thesis aims to identify the main sources of bioavailable N entering the Hawkesbury-Nepean River system.

### *1.3.4 Sources of nitrogen pollution in the Hawkesbury-Nepean River*

In order for water control organisations to formulate an effective management plan, it is vital to be able to identify the main sources of nitrogen and their relative contributions to eutrophication. Excess nitrogen in the Hawkesbury-Nepean River enters the river from a number of sources, primarily run-off from agricultural land and effluent from STPs, but is also contained in storm water, run-off from urban developments, raw sewage or septic tank overflows and industrial wastewater. Nitrogen can also re-enter the water column from sediments under certain conditions. These sources and processes are examined in detail in Chapter 4.

Nutrient run-off from agricultural land and effluent from STPs are virtually identical in their chemical composition (both are mainly in the form of nitrate, with small amounts of ammonium) and are chemically indistinguishable once in the water column (NSW EPA, 1994). The nitrogen stable isotope ratio of each source is markedly different however, and this technique provides a method with which to identify and quantify the sources of nitrogen in an aquatic ecosystem.

## 1.4 Thesis aims and outline.

Understanding the sources of bioavailable nutrients in coastal rivers and estuaries is extremely important. This thesis explores the principle sources of N within a large coastal river and its estuary and develops a new technique, utilising a bioindicator alga, for identifying N sources in this system. Specifically, this thesis aims to refine the techniques used to collect $\delta^{15}N$ data in aquatic ecosystems and then apply this technique to the question of N sources in the Hawkesbury-Nepean river.

The key steps in this thesis are:

1. The development of a bioindicator system: In order to reduce the number of confounding factors potentially masking $\delta^{15}N$ signals in aquatic ecosystems, a "bioindicator" plant, *Microdictyon umbilicatum*, was developed to collect stable isotope data. Details of the literature reviewed, reasons for selecting a "bioindicator" and refinements to the technique are described in Chapter 2.

2. Testing of the bioindicator under a range of environmental conditions: Environmental conditions also exert a significant influence on $\delta^{15}N$ values. The effect of factors such as light, salinity, temperature and nutrient concentrations are investigated (Chapter 3). Those factors that may affect the $\delta^{15}N$ values of the *M. umbilicatum* plants in the field were identified. Corrections could then be made for their influence when interpreting the field results.

3. Using the bioindicator system within a sampling program designed to identify the key sources of nitrogen within the Hawkesbury-Nepean River and estuary: The characteristic $\delta^{15}N$ values of each of the main sources of N contributing to eutrophication in the Hawkesbury-Nepean River were determined (Chapter 4). These values were used to interpret the field results in terms of the main source of bioavailabe N at each site.

4. To explore how seasonality and estuarine flows affect the relative importance of nitrogen sources over time: *M. umbilicatum* plants were used to collect $\delta^{15}N$ data from the Hawkesbury-Nepean River over a three-year period, in order to determine the main sources of N contributing to the eutrophication of the river. These results are presented in Chapter 5 of this thesis.

# CHAPTER 2: TRACING SOURCES OF NITROGEN USING $\delta^{15}N$.

## 2.1 General introduction

Tracing nutrients through an ecosystem or to their source is technically difficult and is achievable by only a small selection of methods. Radioisotopes can be used to label a nutrient input, and the radioactive signal followed through any subsequent steps. However, this process is both expensive and potentially hazardous. Radioisotopes are therefore of little practical use for large-scale ecosystem studies of water column processes, although they are a valuable tool for physiological investigations and have also been used to trace sediment movements. Stable isotope ratios can potentially provide a method of labelling and tracing nutrients through a large ecosystem. Stable isotopes are relatively inexpensive, naturally occurring, and have none of the hazards associated with radioisotopes.

Stable isotopes have been used in ecological studies to determine the main source of nutrients for a particular organism (e.g. Schoeninger and DeNiro, 1983; Jackson and Harkness, 1987; France, 1994; Newell *et al.*, 1995) or to elucidate food web relationships (e.g. McConnaughey and McRoy, 1979; Epstep and Vigg, 1985; Raven, 1990; Yoshioka and Wada, 1994). In addition, stable isotopes such as nitrogen are commonly used to trace the flux of particular nutrients through an organism or ecosystem (e.g. Owens, 1985; Peterson and Howarth, 1987; Lin *et al.*, 1991; Bustamante and Branch, 1996; Lindau *et al.*, 1996). In order to use $\delta^{15}N$ as a tracer in a complex natural ecosystem, the $\delta^{15}N$ signal of the original nutrient input must be isolated from variability caused by other factors. Although some studies have gone some way toward solving these problems (e.g. Costanzo *et al.*, 2001), none have managed to eliminate all other possible sources of variability.

There are two main sources of error that must be overcome in order to successfully use stable isotopes as tracers:

1. Elements are not composed of a constant ratio of stable isotopes
2. Living systems discriminate between isotopes under many conditions (Hauck, 1973).

For the vast majority of $\delta^{15}N$ tracer studies, these problems are significant. Many complex reactions are involved between the source and sink of N, all of which have the potential to affect stable isotope ratios.

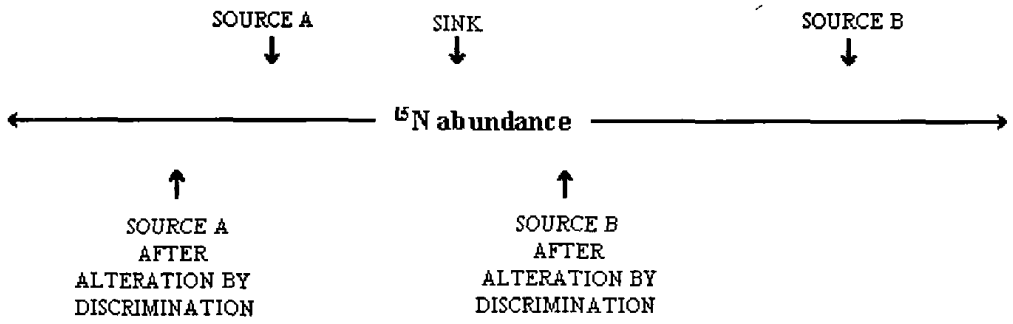
To correctly identify the sources of N in a particular ecosystem using $\delta^{15}N$ data, a number of criteria must be met:

1. The $\delta^{15}N$ values of the source(s) must be known
2. Fractionation(s) occurring between source and sink (the organism being analysed) must be quantified
3. Any fractionation or partitioning of N occurring in the organism itself must be quantified
4. The effects of environmental conditions on these processes must be understood
5. Errors due to processing or analysis of the samples must be allowed for.

It is impossible to measure or estimate many of these effects using conventional methods of $\delta^{15}N$ data collection.

To isolate N source as the cause of differences in $\delta^{15}N$ in an organism collected from a particular ecosystem, it is necessary to isolate this variation from those caused by other factors. The factors affecting $\delta^{15}N$ signals in aquatic ecosystems are discussed in detail in this Chapter. Many of the problems associated with the conventional interpretation of $\delta^{15}N$

data are due to extraneous sources of variation being ignored in the experimental design (Handley and Scrimgeour, 1997). The coupling of manipulative laboratory experiments investigating these sources of variation with appropriate field data can overcome many of these limitations. The effect of discrimination on sink $\delta^{15}N$ values is illustrated in Figure 2.1.




*If fractionation between source and sink is not accounted for, source A appears to be closer in $\delta^{15}N$ value (and hence contribute most N) to the sink. However, if source $\delta^{15}N$ are adjusted for isotopic discrimination, source B is shown to contribute the bulk of N to the sink.*

**Figure 2.1**: Effect of isotopic fractionation when estimating the contribution of two N sources (adapted from Shearer and Kohl, 1993).

Before N sources can be identified or traced through an ecosystem, it is necessary to identify all possible sources of fractionation between the source and sink and to estimate the value of each (Mariotti *et al.*, 1981). This Chapter examines possible sources of isotope discrimination in aquatic ecosystems.

## 2.2 General principles of isotope fractionation

In this thesis, an isotope ratio is defined as the ratio of heavy to light isotopes ($^{15}N:^{14}N$). $\delta^{15}N$ is this ratio relative to a standard of atmospheric N (Section 1.2.1; Equation *1*). Fractionation is a change in the isotope ratio between the reactant (source) and the product (sink). When the difference between source and sink ratios can be calculated, that difference is the discrimination (represented by $\Delta$; after Handley and Scrimgeour, 1997).

$$\Delta = \delta^{15}N \text{ sink/product} - \delta^{15}N \text{ source} \qquad (2)$$

The $\alpha$-value is also used to express discrimination (Handley and Scrimgeour, 1997). $\alpha$ is the relative "reactivities" of $^{14}N$ and $^{15}N$ (see below). A value of $\alpha > 1$ indicates that the product is $^{15}N$-depleted relative to the substrate. For example, an $\alpha$ value of 1.010 is equivalent to a discrimination of –10‰.

$$\delta^{15}N = (\alpha - 1) \times 10^3 \qquad (3)$$

Because of the slight differences in mass between isotopes of the same element, differences in certain physico-chemical properties are seen for each isotope. For example, bonds formed by the light isotope are weaker than those involving the heavy isotope. Hence, during a chemical reaction, molecules containing the light isotope will react slightly more readily than those containing the heavy isotope (Kaplan, 1975; Hoefs, 1997). Generally, however, non-biological chemical reactions do not significantly discriminate against either isotope, apart from a few notable exceptions such as evaporation/condensation reactions (Heaton, 1986; Hoefs, 1997). For example, $NO_3^-$ and $NH_4^+$ in fertilisers are produced by quantitative processes resulting in little overall fractionation, and therefore have $\delta^{15}N$ values close to zero (Heaton, 1986).

Both physico-chemical (e.g. the preferential loss of the lighter isotope to the gaseous phase) and biochemical (e.g. denitrification) processes can fractionate $^{15}N/^{14}N$. Different patterns of fractionation can occur, depending on whether the reaction involved is an equilibrium reaction (e.g. the pH dependent balance between $NH_4^+$ and $NH_3$ forms of N) or a kinetic one (e.g. an enzyme-mediated reaction; Handley and Scrimgeour, 1997; Hoefs, 1997).

### *2.2.1 Equilibrium reactions*

Equilibrium constants are determined by differences in the structure and energy of two compounds at equilibrium (Zumdahl, 1989). These parameters are affected by the isotopic composition of the compounds involved (Shearer and Kohl, 1993). For example, the equilibrium isotope effect causes $^{15}N$ to be less abundant in $NH_3$ than in $NH_4^+$. Volatilisation of $NH_3$ therefore enriches the remaining $NH_4^+$ in $^{15}N$ and raises the $\delta^{15}N$ of soils where this occurs (Shearer and Kohl, 1993).

### *2.2.2 Kinetic isotope effects*

Differences in the reaction rate of molecules containing $^{14}N$ and $^{15}N$ result in differences in the abundance of each isotope in the product compared to the substrate (Shearer and Kohl, 1993).

Each step in a reaction has an intrinsic isotope effect, which is the difference in the rate constant between molecules containing $^{14}N$ and those containing $^{15}N$. Although the magnitude of intrinsic isotope effects decreases with increasing temperature, within the range of physiological conditions this variation is minimal, and intrinsic isotope effects can in most cases be dismissed as trivial (Shearer and Kohl, 1993).

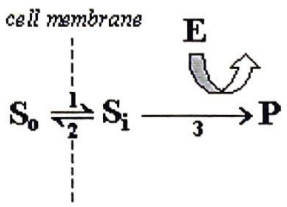
The overall isotope effect for an entire reaction depends on the magnitude and direction of the intrinsic effect for each individual step, their relative rates and the mechanism of the reaction (Heaton, 1986; Shearer and Kohl, 1993).

Generally, the passage of a molecule into a biological system might follow the steps:

1. Diffusion of the substrate to the cell
2. Transport or diffusion of the substrate into the cell
3. Association of the substrate with an enzyme
4. Catalysis (in one or more steps)
5. Release of the product from the enzyme.

Most of these steps are non-discriminatory. There is no isotope effect associated with diffusion through an aqueous medium (Shearer and Kohl, 1989). Transport through the membrane (both active and passive) is usually non-discriminatory in practice (Handley and Scrimgeour, 1997). Enzyme substrate association and release of the product also shows no discrimination against either isotope. In general, it is the catalytic step that fractionates (Macko *et al.*, 1986; Shearer and Kohl, 1993; Handley and Scrimgeour, 1997). For example, during the *in vivo* transfer of N from glutamic acid to aspartic acid, $^{14}NH_2$ was found to react 1.0083 times faster than $^{15}NH_2$ (Macko *et al.*, 1986).

The relative rates of steps in a reaction sequence may vary with environmental conditions and the concentration of molecules entering the reaction at different steps (Shearer and Kohl, 1993). This in turn affects the final amount of fractionation between the substrate and the product (Figure 2.2).




*The nitrogen-containing medium outside the cell ($S_0$) enters the cell in step 1. Inside the cell, the substrate ($S_i$) is metabolised by the enzyme (E) in step 3. Unreacted substrate exits from the cell in step 2. The $^{15}N$ abundance of the substrate $S_i$ is increased by step 3, which discriminates against molecules containing $^{15}N$. As $S_i$ exits from the cell, it enriches the external substrate pool in $^{15}N$. The degree of expression of the fractionation associated with step 3 depends on the rate of step 2 relative to the rate of step 3.*

**Figure 2.2**: A model for an enzyme-catalysed reaction (adapted from Shearer and Kohl, 1993).

### *2.2.3 The effect of open and closed systems*

The final influence on fractionation is whether the reactions take place in a closed or open system. Although there are no completely closed systems in nature, in terms of isotopes, some systems behave as if they were closed. This is because the rate of substrate replenishment is slow relative to the rate at which the reaction proceeds. Generally, in a closed system, the reaction will initially use the lighter isotope (Shearer and Kohl, 1993; Hoefs, 1997). However, as the reaction proceeds, more of the remaining heavy isotope is converted to product. If the reaction goes to completion, there will be no net fractionation, as all the isotopes, both heavy and light, will have moved from substrate to product (Shearer and Kohl, 1993; Handley and Scrimgeour, 1997). Many enzyme reactions have large potential

fractionations which are never fully expressed on the level of the analysed sample because the substrate is limited or the remaining substrate (which is enriched in $^{15}N$) is not lost from the system being analysed (Shearer and Kohl, 1989). For example, the enzyme GS has a potential fractionation of –17‰ (Yoneyama *et al.*, 1993) but plant $\delta^{15}N$ values of below –7‰ are seldom reported, mainly due to substrate limitation effects (Handley and Scrimgeour, 1997).

In contrast, an open system, as is typical of most natural ones, has an effectively infinite supply of substrate relative to the demands of the reaction (Handley and Scrimgeour, 1997). This means that the reaction is able to preferentially use the lighter isotope from a constantly renewed pool, and is therefore never "forced" to convert the heavier isotopes to product. Any fractionation is therefore fully expressed.

## 2.3 Sources of $\delta^{15}N$ variation in ecosystem studies

As outlined in the previous Section, biological reactions often discriminate against one isotope, usually the heavier one (Hoefs, 1997). In living organisms, significant variation in $\delta^{15}N$ values can be related to the trophic level of the organism or genetic variation between individuals, which may result in differences in isotope uptake or assimilation. Ultimately, these are driven by variation in the component processes in each biological system. The selection of a particular part of an organism for analysis, and the analysis itself, can also introduce error (Handley and Scimgeour, 1997).

### *2.3.1 Trophic level*

In ecological studies, nitrogen stable isotope ratios are often assumed to indicate the trophic level of an organism (Handley and Scrimgeour, 1997). Many studies have analysed the $\delta^{15}N$ of organisms and used these data to elucidate food webs in a variety of ecosystems (e.g. Bunn and Boon, 1993; France, 1994; Newell *et al.*, 1995; Kikuchi and Wada, 1996). General patterns, showing a progressive increase of $\delta^{15}N$ with trophic level, have emerged.

The average change in $\delta^{15}N$ between trophic levels for a number of ecosystems is estimated to be 3.2‰ (Peterson and Fry, 1987). In addition, consumers in marine ecosystems have been calculated to be systematically heavier than the $\delta^{15}N$ values of their diets by 3.4 ± 1.1 ‰ (Wada and Hattori, 1991). However, these patterns are not always consistent, and may mask a number of complex internal processes within ecosystems.

Differences in $\delta^{15}N$ between trophic levels are obviously useful if the aim of a study is to describe food web patterns (e.g. Spies *et al.*, 1989; Boon and Bunn, 1994; Yoshioka and Wada, 1994; Peterson *et al.*, 1995). However, in the case of "tracer" studies, an organism's trophic level may be a source of error when interpreting $\delta^{15}N$ data. Unfortunately, identifying the trophic level of organisms is not always easy, and the exact fractionation of $\delta^{15}N$ by each species is usually only estimated (Bustamante and Branch, 1996). Considerable variation in the amount of fractionation from food source to consumers of the same trophic level exists (Peterson et *al.*, 1995; Yamamuro and Minagawa, 1995; Kikuchi and Wada, 1996). For example, Peterson and Howarth (1987) found that $\delta^{15}N$ values increased by approximately 4‰ from the food source in brook trout, but in the same feeding experiment found an increase of only 1.6‰ from the food source in gypsy moth larvae. This is not surprising, given the widely different biochemical pathways for the uptake and assimilation of N between fish and insects. This study does, however, illustrate the need for caution when interpreting $\delta^{15}N$ data

in food web studies. Vanderklift and Ponsard (2003) reiterate this need for caution in their work on the sources of variation in consumer-diet $\delta^{15}N$ studies. In studies seeking to identify the fate of a nutrient input (e.g. sewage; Van Dover *et al.*, 1992, Costanzo *et al.*, 2001), the trophic level of organisms selected for analysis is of even greater importance, as $\delta^{15}N$ variation between organisms has the potential to mask small differences in $\delta^{15}N$ between N sources.

### *2.3.2 Genetic variation*

Significant differences due to genetic variation can exist between species (even those from the same order) in the extent to which N is fractionated during uptake and assimilation. This can lead to differences in the $\delta^{15}N$ values of tissues due to biochemical variation between individuals and not to other, external, factors. For example, it was found that the isotopic composition of deposit-feeding polychaetes was species-specific (Peterson and Howarth, 1987; Kikuchi and Wada, 1996).

Genetic variability between individuals, particularly in wild populations, may result in differences in the enzymatic pathways used to take up and metabolise N compounds. In turn, this can cause variation in the final stable isotope ratio of individuals. Studies on the effect of genetic variability within a single species on $\delta^{15}N$ values have not been specifically carried out, but at least some of the significant variation between individuals in many studies may be attributed to genetic differences (e.g. Bunn and Boon, 1993; Newell *et al.*, 1995; Bustamante and Branch, 1996). In plant-based studies, vegetation type, species and plant functional type can all affect $\delta^{15}N$ signals, yet are often ignored when interpreting data (Handley and Scrimgeour, 1997).

### *2.3.3 Sample selection*

The selection of sample material from an organism is extremely important, as each organ may assimilate, fractionate or store N in a different manner (Ledgard *et al.*, 1985; Yoneyama, 1995; Evans *et al.*, 1996; Handley and Scrimgeour, 1997; Vanderklift and Ponsard, 2003). This can lead to marked differences in $\delta^{15}N$ between organs. For example, Yoneyama and Kaneko (1988) found the difference in $\delta^{15}N$ between komatsuna plant leaves and roots to be up to 6.9‰.

If the isotope ratio of the whole organism is not measured, samples must be carefully selected and consistent. Samples must not be interpreted to reflect the overall $\delta^{15}N$ of the individual. Possible errors associated with sample selection in plants are further discussed in Sections 2.4.2 and 2.4.3.

### *2.3.4 Processing methods and analysis errors*

Differences in processing methods or errors in the analysis itself can also contribute to erroneous stable isotope values. Guidelines and a review of methods for the preparation of samples to be analysed for $\delta^{15}N$ are outlined by Ehleringer and Rundel (1989) and Shearer and Kohl (1993).

The following possible sources of error were identified:

1. Fractionation during sample preparation: isotope fractionations can be associated with incomplete conversion of N from one form to another or with N loss.
2. Contamination: care must be taken not to contaminate natural samples with $^{15}N$ enriched samples. As natural samples have extremely low total amounts of $^{15}N$, even trace amounts of contamination may significantly change $\delta^{15}N$ values.
3. Non-representative sub-samples: obtaining a representative sub-sample can be difficult with non-homogeneous samples such as soils, plant or animal material.
4. Systematic or measurement errors: this possibility should be minimised by replicate samples or standards being included in all sample sets.
5. Inconsistent sample N conversion to $N_2$ gas: the mass spectrometer measures only $N_2$ gas. The conversion between sample N (in whatever form) and $N_2$ must be as consistent as possible.

Sample preparation has become semi-automated in recent years and improvements to both methodology and equipment have meant that precision in N sample processing in most commercial laboratories is in the order of 0.1 – 0.2 ‰ (Shearer and Kohl, 1993; S. Howe, University at Albany, New York, pers. comm.). Error caused by processing and analysis is therefore negligible in most cases, particularly in comparison to that due to experimental design flaws.

## 2.4 Sources of $\delta^{15}N$ variation in plants and algae

In this study, the central organism used to assess $\delta^{15}N$ variation in the environment is a green alga, *Microdictyon umbilicatum*. Plants are particularly sensitive to the influence of their physical environment on the uptake, assimilation and partitioning of nitrogen compounds. All of these steps have the potential to vary fractionation of N. Light levels, temperature, pH and the concentration and form of nitrogen compounds available to the plant can all affect the amount and direction of fractionation in plants (Losada *et al.*, 1987; Ullrich, 1987; Wallace, 1987; Syrett, 1988; Pilbeam and Kirkby, 1992; Thornton *et al.*, 1995; Evans *et al.*, 1996). In addition, the rate of exchange between the plant and its environment, and the partitioning of nitrogen compounds into different plant organs can also cause large differences in $\delta^{15}N$, depending on the part of the plant analysed and even the time of day at which it is sampled (Handley and Scrimgeour, 1997).

### *2.4.1 Assimilation of nitrogen compounds in algae and higher plants*

The assimilation of nitrogen compounds is essential for all plants. The two major forms of nitrogen taken up by plants are ammonium ($NH_4^+$) and nitrate ($NO_3^-$) ions. Nitrate is the main form of N available to plants in both aquatic and terrestrial ecosystems, and most plants have adapted to take up $NO_3^-$ as well as $NH_4^+$, despite $NO_3^-$ being more energetically expensive to assimilate (Raven *et al.*, 1992). Assimilation of all nitrogen compounds is aided and regulated by a series of enzymes, which can differ between groups and even species of plants (Syrett, 1988). However, in general, the main differences in biochemistry are between algae and higher plants.

*Ammonium uptake*

In green algae, $NH_4^+$ is taken up through the membrane by diffusion, converted to glutamine and finally reduced to glutamate (Streicher and Valentine, 1973; Beeves, 1976; Ullrich, 1987; Syrett, 1988). Each of these stages is catalysed by enzymes (Figure 2.3).

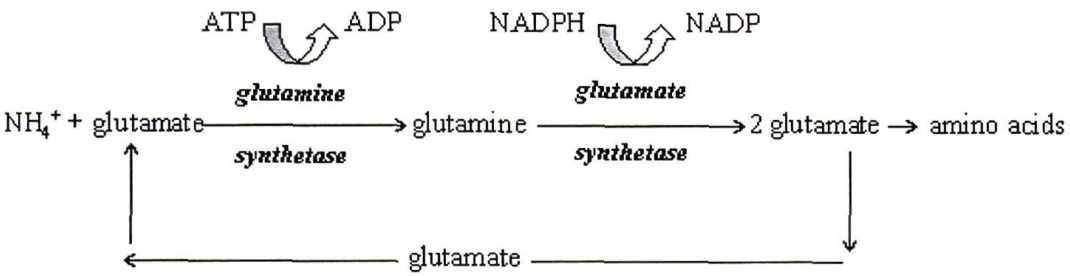



**Figure 2.3**: Enzymes involved in the uptake of ammonium in algae

There are two main isoforms of glutamine synthetase (GS) in algae. The first is generally found in the cytoplasm, and the second in the chloroplasts (Vega *et al.*, 1987; Syrett, 1988). Some species of algae have both forms of the enzyme, others have only one (Syrett, 1988). There are also two forms of glutamate synthetase, one that uses NADH as a reductant and a rarer form that uses ferredoxin (Ullrich, 1987; Vega. *et al.*, 1987; Atwell *et al.*, 1999). The isoform associated with NADH is found in the cytoplasm and is largely responsible for the initial assimilation of $NH_4^+$, while the other appears to play a role only in the reassimilation of $NH_4^+$ released during photorespiration (Syrett, 1988).

In the leaves of higher plants, the reduction of glutamine is carried out by glutamate synthase or glutamate dehydrogenase instead of glutamate synthetase (Beeves, 1976; Raven *et al.*, 1999). Ferrodoxin and not NADPH is the electron donor in this case (Figure 2.4).

However, assimilation of $NH_4^+$ in the roots of higher plants follows a similar pathway to that in algae, with NADPH or NADH replacing ferredoxin in the pathway (Raven *et al.*, 1999). This difference, and its relationship with photosynthetic tissue, is discussed further in Section 2.4.4.

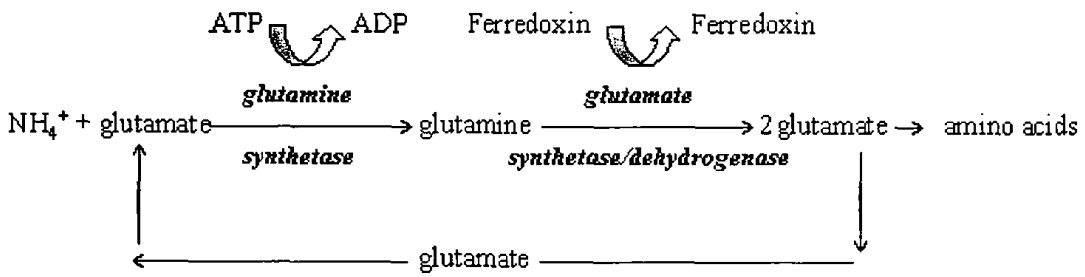



**Figure 2.4**: Enzymes involved in the uptake of ammonium in green plants.

*Nitrate uptake*

If $NO_3^-$ is taken up by a plant or algae, it is reduced to $NO_2^-$ and then to $NH_4^+$, where it is fed into the normal $NH_4^+$ assimilation pathway of the organism (Ledgard *et al.*, 1985; Vega *et al.*, 1987; Syrett, 1988; Yoneyama *et al.*, 1998). In higher plants, reduction to $NH_4^+$ takes place most commonly in the leaves (Beeves, 1976; Mengel and Kirkby, 1982). Again, these steps are all catalysed by enzymes (Figure 2.5).

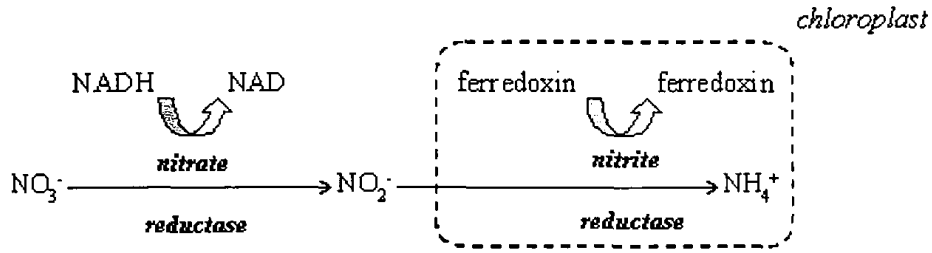



**Figure 2.5**: Enzymes involved in the uptake of nitrate in green plants and algae.

In leaves, nitrate reductase (NR) is found in the cytoplasm, but is often near or associated with the chloroplast, which contains the nitrite reductase (NiR; Mengel and Kirkby, 1982; Wallace, 1987; Wallsgrove, 1987). The $NH_4^+$ produced by this pathway remains in the chloroplast, where it is reduced by the GS GOGAT cycle.

Non-green tissue (roots) can also assimilate $NO_3^-$ in the cytoplasm. In this case, glycolysis provides the NADH needed for reduction to $NO_2^-$ (Beeves, 1976). The mechanisms generating NADH in roots and leaves are discussed further in Section 2.4.4.

In some algae, NADPH as well as NADH may function as a source of reducing power for nitrate reductase. NADPH-dependent nitrate reductase has also been reported in some higher plants, but is not as common as the NADH powered pathway (Mengel and Kirkby, 1982; Atwell *et al.*, 1999).

Any isotopic fractionation associated with the uptake of $NO_3^-$ occurs at the step catalysed by NR (Ledgard *et al.*, 1985; Wallace, 1987; Wallsgrove, 1987; Yoneyama *et al.*, 1998). This fractionation can be up to –15‰ in plants and algae (Wada and Hattori, 1991; Yoneyama *et al.*, 1998). Some fractionation is theoretically possible at the GS step in the assimilation of

$NH_4^+$, but in practice, the efficiency of the process leaves very little unassimilated $NH_4^+$, therefore resulting in negligible fractionation (Evans *et al.*, 1996; Yoneyama *et al.*, 1998; Section 2.2.3).

Many other factors interact with these enzyme-catalysed pathways, in particular, light levels, concentration and the form of nitrogen available. These are discussed in the Sections below and are experimentally explored as far as influences on the bioindicator system developed in this thesis.

### *2.4.2 Concentration and form of nitrogen: ammonium and nitrate*

Ammonium and nitrate are taken up by both plants and algae. Despite being the more common of the two forms, the uptake of $NO_3^-$ is depressed in higher plants when both $NH_4^+$ and $NO_3^-$ are present. $NH_4^+$ reduces the synthesis of the carrier responsible for $NO_3^-$ uptake, or inhibits the enzymes responsible for the assimilation of $NO_3^-$ in the plant (Pilbeam and Kirkby, 1992; Ullrich, 1987; Syrett, 1988, Raven *et al.,* 1999). In most cases, the activity of nitrate reductase is increased by the presence of $NO_3^-$, and decreased by the presence of $NH_4^+$ (Mengel and Kirkby, 1982). In higher plants, $NH_4^+$ uptake is not affected by $NO_3^-$ concentration, but $NO_3^-$ uptake can be competitively depressed by $NH_4^+$ (Mengel and Kirkby, 1982). Most algae also take up $NH_4^+$ preferentially and in some cases, $NO_3^-$ is not used at all until all $NH_4^+$ ions have disappeared (Morris, 1974). For marine microalgae, the uptake of $NO_3^-$ is completely inhibited by $NH_4^+$ concentrations in excess of 2 mmol.m $^{-3}$ (Raven *et al.*, 1992).

Differences in the concentration and ratio of $NH_4^+$, $NO_3^-$ and other N compounds available to the plant may result in significant fractionation of N between source and plant. In general, the amount of discrimination that takes place is often positively correlated with the concentration of $NO_3^-$ in the growth medium (Kohl and Shearer, 1980; Mariotti *et al.*, 1981; Bergersen *et al.*, 1988; Evans *et al.*, 1996; Table 2.1).

**Table 2.1**: Isotope fractionation for N assimilation (adapted from Hoch *et al.*, 1992; Fogel and Cifuentes, 1993).

| **Type of N assimilation** | **Resulting fractionation Δ** |
|---|---|
| ***N fixation*** | -3 to +1 ‰ |
| ***$NH_4^+$ assimilation*** | |
| *millimolar concentrations* | 0 to –15‰ |
| *micromolar concentrations* | -3 to -27 ‰ |
| ***$NO_3^-$ assimilation*** | |
| *millimolar concentrations* | 0 to –24‰ |
| *micromolar concentrations* | -4 to -10‰ |

The assimilation of $NO_3^-$ is mediated by an energy-requiring transport process and the consecutive action of the enzymes nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase (Vega *et al.*, 1987). Substantial fractionation may occur when $NO_3^-$ is the sole or primary source of nitrogen. Discrimination is usually positively correlated with the concentration of $NO_3^-$ available to the plant (Bergersen *et al.*, 1988; Evans *et al.*, 1996). Relatively high external $NO_3^-$ concentrations can produce fractionation of up to 24‰ (Table 2.1). In contrast, low concentrations rarely produce any discrimination (Ullrich, 1992; Evans *et al.*, 1996). At low concentrations, $NO_3^-$ is taken up by an active transport system, which

produces little fractionation. Higher concentrations of $NO_3^-$ are assimilated by a discriminatory enzyme system, which at times produces significant fractionation (Evans *et al.*, 1996). In addition, low concentrations of $NO_3^-$ effectively act as a closed system, while high concentrations mimic an open system (see Section 2.2.3).

Changes in fractionation associated $NH_4^+$ concentration are generally correlated with a switch in the pathway of $NH_4^+$ uptake. At millimolar concentrations of $NH_4^+$, $NH_3$ diffuses through the membrane, where it is transformed to $NH_4^+$ and assimilated by glutamate dehydrogenase, an enzyme with preferentially assimilates $^{14}N$. This produces significant fractionation. At micromolar concentrations of $NH_4^+$, uptake is achieved via active transport of the $NH_4^+$ ions, and assimilated via glutamine synthetase (Ledgard *et al.*, 1985; Hoch *et al.*, 1992). Because all the available nitrogen will be effectively used by the reaction, little fractionation arises from this step (mimicking a closed system; Section 2.2.3).

Nitrogen may also be distributed differently within the plant, depending on the form of N in the source. For example, N from $N_2$ gas and $NH_4^+$ was found to be distributed preferentially to the developing organs, young leaves and developing pods of soybeans and rice. In comparison, N supplied in the form of $NO_3^-$ was actively distributed to both developing and mature plant organs (Yoneyama and Kumazawa, 1972; Yoneyama and Ishizuka, 1982). In some studies, shoots and leaves were found to be enriched in $^{15}N$ compared to the plants' roots, while in other studies this pattern was reversed (Bergersen *et al.*, 1988; Yoneyama and Kaneko, 1988). More recent studies have failed to resolve a mechanism for this variation, but all have found the source of nitrogen ($NH_4^+$ or $NO_3^-$) strongly influenced intra-plant patterns of $\delta^{15}N$ (reviewed in Evans *et al.*, 1996).

Because of this, each plant organ does not equally reflect the $\delta^{15}N$ of each form of N supplied to the plant (see also Sections 2.3.3 and 2.4.3). It is therefore important to carefully select plant organs for analysis, particularly if the aim is to distinguish between sources with different ratios of $NH_4^+$: $NO_3^-$.

As well as $NH_4^+$ and $NO_3^-$, plants may directly use many forms of combined N (e.g. urea, purines, amino acids, amines) with or without symbiotic assistance (Raven *et al.*, 1993). Each of these compounds may have a distinct $\delta^{15}N$ signal, and their uptake and assimilation (with any associated fractionation) may affect the overall $\delta^{15}N$ of the plant. The effect of N concentrations on the final $\delta^{15}N$ of *M. umbilicatum* is discussed in Chapter 3.

### *2.4.3 Rate of exchange with the environment*

The rate of exchange between an organism and the environment can significantly affect its final $\delta^{15}N$ signal. Many organisms can retain nitrogen compounds for long periods of time, which may range up to several years (Pate, 1980; Atwell *et al.*, 1999). These compounds reflect the nitrogen environment of the organism at the time the compound was formed, and any fractionations that have occurred during the formation, transport or storage of the compound (Shearer and Kohl, 1989; Handley and Scrimgeour, 1997).

Nitrogen turnover in plants can be characterised by three main steps (Figure 2.6; Mengel and Kirkby, 1982; Atwell *et al.*, 1999). All three of these fractions are influenced by plant nutrition, environmental conditions and in particular by the supply of N.

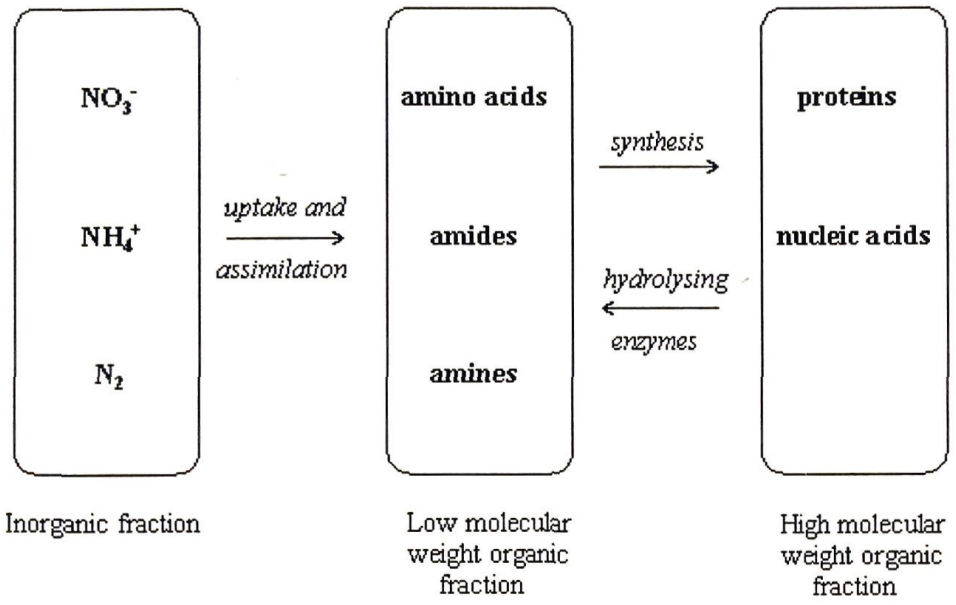



**Figure 2.6**: Nitrogen turnover in plants (adapted from Mengel and Kirkby, 1982).

Plants may store nitrogen in a soluble form, in amounts of up to 5% of total tissue dry weight, in the fleshy tissues of stems, bark or storage organs (Pate, 1980). This pool of nitrogen dilutes any new uptake and reflects the balance of the $\delta^{15}N$ of the remaining and new nitrogen compounds (Handley and Scrimgeour, 1997). If stored compounds make up a large portion of the total nitrogen content of an organism, this may significantly alter its overall stable isotope ratio. If whole plant $\delta^{15}N$ is not measured, the selection of storage organs as samples may also introduce considerable error (see Section 2.4.3).

In "tracer" studies, this effect can be particularly problematic, as organisms may not reflect their current N environment, but those of their past (Shearer and Kohl, 1989). Together with those sources of error previously discussed in this Chapter, the nitrogen "history" of an organism can confound $\delta^{15}N$ experiments seeking to determine nitrogen sources. Turnover rates of N in *M. umbilicatum* under various environmental conditions are presented in Section 3.3. An internal control to ensure N turnover was complete in the algae was included in all field experiments and is further discussed in Section 5.2.

### *2.4.4 Light*

Most plants and algae assimilate nitrogen compounds more rapidly in the light than in the dark (Morris, 1974; Atwell *et al.*, 1999). Both the intensity and quality of light may affect the uptake of nitrogen by modulating either the uptake or reduction of compounds (Beeves, 1976; Mengel and Kirkby, 1982; Losada *et al.*, 1987; Wallace, 1987).

Nitrate and $NO_2^-$ reduction in green organisms can be considered a basic photosynthetic process as it uses, either directly or indirectly, a large proportion of the total reducing power generated in the chloroplast (Maldonado and Aparicio, 1987). The photochemical reactions of photosynthesis (Photosystems I and II) produce two fundamental components for plant growth:

1. chemical energy (as ATP)
2. the electrons required to reduce oxidised forms of elements to forms able to be assimilated.

In the chloroplast, electrons generated by the splitting of water molecules by photosystem II are passed via P680+ (a pair of chlorophyll a molecules) and a number of electron transport cofactors to photosystem I. Electrons then pass through a number of iron-sulphur centres out of photosystem I and finally to ferredoxin (Kamin and Privalle, 1987; Atwell *et al.*, 1999). Ferredoxin can then pass electrons to ferredoxin-NADP reductase, which then produces NADPH. Ferredoxin or NADPH may then act as electron donors for the reduction and assimilation of nitrogen compounds by enzymes (Figure 2.4; Losada *et al.*, 1987; Atwell *et al*, 1999). The two photosystems are juxtaposed across the thylakoid membranes in such a way that linear electron transport can also be harnessed for charge separation. This leads to an accumulation of $H^+$ ions within the lumen of illuminated thylakoids which can then be used for ATP generation (Atwell *et al.*, 1999).

It was originally speculated that $NO_3^-$ utilisation in higher plants was directly affected by light levels due to the reduced production of reductants by photosynthesis under low illumination (Morris, 1974; Losada *et al.*, 1987; Maldonado and Aparico, 1987). However, the reductant for $NO_3^-$ reduction is usually NADH, while the primary product of photosynthesis is NADPH, which is also largely contained in the chloroplast (Figure 2.7; Beeves, 1976; Mengel and Kirkby, 1982). It seems more likely that $NO_3^-$ assimilation is linked to light levels indirectly via the production of sugars in the chloroplast during photosynthesis. These sugars then cross the chloroplast membrane to the cytoplasm, where they are metabolised in glycolysis. Glyceraldehyde-3-phosphate produced by glycolysis (or produced directly by photosynthesis) is oxidised to phosphoglyceric acid, which concurrently reduces NAD to NADH (Figure 2.7; Klepper *et al.*, 1971; Beeves, 1976; Mengel and Kirkby, 1982; Atwell *et al.*, 1999; Raven *et al.*, 1999). Some of this generated NADH may then furnish the electrons to reduce $NO_3^-$ to $NO_2^-$ (Klepper *et al.*, 1971). NADH may also be indirectly supplied by the chloroplast via the oxaloacetate-malate shuttle (Mengel and Kirkby, 1982). Oxaloacetate is taken up by the chloroplast and reduced by NADPH (from photosynthesis) to malate. This is exported to the

cytoplasm and oxidised to oxaloacetate again, with the result of reducing NAD to NADH (Figure 2.7; Mengel and Kirkby, 1982; Raven *et al.*, 1999). Roots can also assimilate $NO_3^-$, and the production of NADH during gylcolysis provides a mechanism to provide the electrons needed to reduce $NO_3^-$ in non-photosynthetic tissue (where NADPH is not produced).

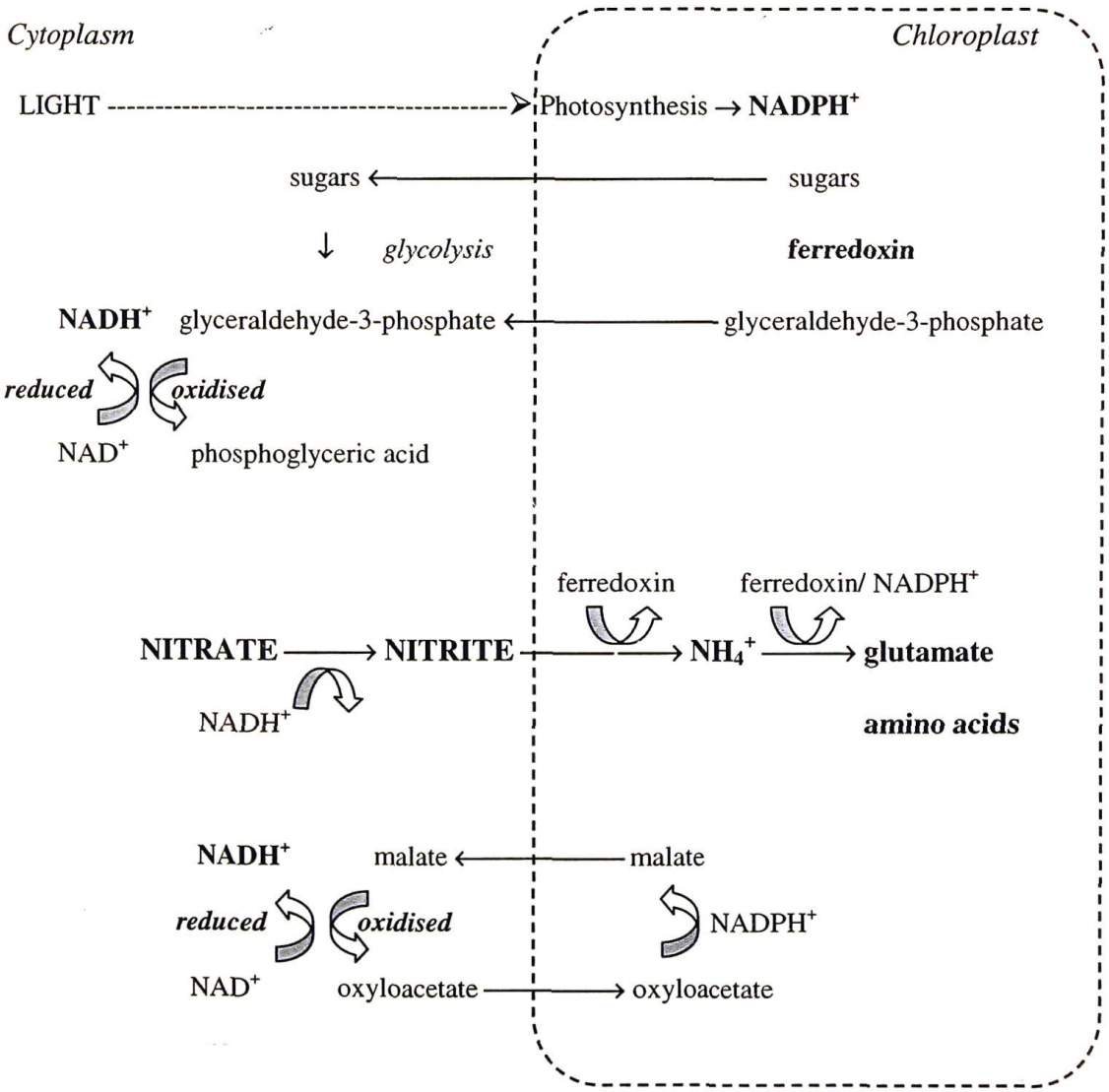



**Figure 2.7**: The contribution of light to the reduction of nitrogen compounds by plant enzymes.

In algae, nitrate reductase is commonly supplied with electrons by NADPH. In this case, light levels will directly affect the rate of $NO_3^-$ assimilation via the photosynthetic production of NADPH, and the reaction will occur in the chloroplast (Mengel and Kirkby, 1982; Losada *et al.*, 1987; Wallace, 1987; Raven *et al.*, 1999). In algae which utilise NADH as an electron donor, illumination will affect the rate of $NO_3^-$ reduction via the indirect pathway of glycolysis, as discussed above for higher plants.

The assimilation of $NH_4^+$ in some algae also appears to be light dependent. For example, the assimilation of $NH_4^+$ in the green alga *Chlamydomonas* occurs through a light-dependent GS-GOGAT cycle (Vega *et al.*, 1987). However, this is not a consistent trend for all algae, but seems to depend largely on the isoform of GS present, the overall enzyme pathway used to assimilate $NH_4^+$ and on the prevailing environmental conditions (Ullrich, 1987; Vega *et al.*, 1987; Wallsgrove, 1987; Atwell *et al.*, 1999).

Nitrite reduction in leaves and some green algae is also accelerated in the presence of light (Beeves, 1976; Mengel and Kirkby, 1982). This is because ferredoxin carries the electrons produced by photosynthesis to the nitrite reductase. However, this does not explain findings of nitrite reductase in root proplastids or the ability of roots to assimilate $NO_2^-$ (Beeves, 1976; Mengel and Kirkby, 1982; Atwell *et al.*, 1999). Light can also affect the rate, order and amount of $^{15}N$ incorporated into various amino acids in algae and higher plants (Cooper and DeNiro, 1989). In particular, the transamination of glutamine has been found to be directly controlled by light (Ito and Kumazawa, 1978).

At high light intensities, discrimination against either N isotope is unlikely (Shearer and Kohl, 1989). However, if light levels are restricted, discrimination may occur as the enzymatic assimilation of N is now the rate-limiting step (see Section 2.2.2 and Figure 2.2).

For many aquatic algae, the main changes to light availability will be caused by depth. Turbid waters can reduce light penetration significantly, and for many species, this is one of the limiting factors to survival in these areas (Cooper and DeNiro, 1989; NSW EPA, 1994). The effect of light levels on the final $\delta^{15}N$ of algae and higher plants must be taken into account if they are likely be significantly different between samples. This commonly occurs where samples are taken from different sites in a river such as the Hawkesbury-Nepean, which has widely varying turbidity and hence light penetration (see Section 1.3.2). Results of experiments on the effect of light levels on the $\delta^{15}N$ of *M. umbilicatum* are presented in Section 3.5.

### *2.4.5 Temperature*

In general, chemical reactions proceed at a faster rate at higher temperatures (Zumdahl, 1989). However, biological reactions are mediated by enzymes, protein catalysts that work only in narrow, specific temperature ranges. Hence, biological reactions generally proceed fastest at an optimal temperature specific to each organism, usually close to the median ambient temperature of the organism's usual environment (Atwell *et al.*, 1999). Temperatures outside the optimum range can greatly reduce growth rates, reproductive capability and metabolic efficiency of organisms (Troshin, 1967; Prosser, 1991). The growth of algae is more likely to be nutrient-limited at higher temperatures. Maximal growth also occurs at higher temperatures (Sterner and Grover, 1998). The temperature range for active growth of higher plants and algae is generally constrained to between 5 and 45°C (Atwell *et al.,* 1999).

Nitrogen uptake and metabolism in plants is not grossly mediated by temperature, however, temperature may affect nitrogen uptake and growth rates, which in turn influence nitrogen turnover, storage and demand (O'Donahue and Dennison, 1997; Vaast *et al.,* 1998; Sakamoto

and Bryant, 1999). Plant enzymes are affected by temperature fluctuations, and generally have a narrow thermal kinetic window of only 8-25°C (Atwell *et al.*, 1999). At temperatures outside this optimum, plants may eventually adjust key enzyme reactions to a new optimum, but will suffer thermal stress until this occurs. The most extreme examples of this are seen in northern hemisphere deciduous plants, where nitrogen storage and transport is greatly affected by seasonal temperature fluctuations (Thornton *et al.*, 1995; Handley and Scrimgeour, 1997). The uptake of $NH_4^+$ and $NO_3^-$ is also directly temperature–dependent in some plant species, with rates of uptake of both ions being depressed at lower temperatures (Clarkeson and Warner, 1979; Mengel and Kirkby, 1982). Temperature generally has a greater effect on the uptake of $NO_3^-$ than of $NH_4^+$ (Clarkeson and Warner, 1979; Cruz C. Lips and Martins-Loucao, 1993; Vaast *et al.*, 1998). Some algal species can not actively take up any $NO_3^-$ or $NO_2^-$ at temperatures below 15°C (Sakamoto and Bryant, 1999). The reason for this pattern is not clear, although it has been suggested that $NH_4^+$ and $NO_3^-$ are taken up at spatially distinct sites in the plant membrane and that the region where nitrate is taken up is more temperature-sensitive (Clarkeson and Warner, 1979; Mengel and Kirkby, 1982; Vaast *et al.*, 1998).

Any changes in nitrogen uptake may in turn influence the fractionation and turnover of nitrogen compounds, and hence the final $\delta^{15}N$ values of the plant. Results of experiments on the effect of temperature on the $\delta^{15}N$ of *M. umbilicatum* are presented in Section 3.6.

### *2.4.6 pH and salinity*

As well as mediating the equilibrium reaction between $NH_4^+$ and $NO_3^-$

$$NH_4^+ + OH^- \longleftrightarrow NO_3^-(aq) + H^+ \qquad (4)$$

pH can also influence the uptake of either ion into the plant. Ammonium is taken up best in a neutral pH, and uptake is depressed as pH falls. The converse is true for $NO_3^-$, which is taken up more rapidly at low pH values (Mengel and Kirkby, 1982; Raven *et al.,* 1999). For example, at pH 4.0 the uptake of $NO_3^-$ into barley seedlings was much higher than the uptake of $NH_4^+$, while at pH 6.8, the uptake of each ion was approximately the same (Mengel and Kirkby, 1982; Pilbeam and Kirkby, 1992).

It has been suggested that the reduction of $NO_3^-$ uptake at high pH may be due to the competitive effect of $OH^-$ ions suppressing the $NO_3^-$ transport system (Mengel and Kirkby, 1982). Also, $NH_4^+$ in solution can be toxic to plant growth, possibly triggering a switch to $NO_3^-$ assimilation if $NH_3$ *(aq)* levels become too high (Mengel and Kirkby, 1982; Atwell *et al.*, 1999; Raven *et al.*, 1999). At acidic pHs, many plant species can tolerate much greater levels of $NH_4^+$, as the presence of $H^+$ ions suppresses the production of $NH_3$ *(aq)* (see Equation 4).

pH is often relatively consistent in aquatic environments and any significant changes are generally directly correlated with salinity. This trend is also true for the Hawkesbury-Nepean River (NSW EPA, 1994). Salinity itself also influences the uptake of nitrogen compounds into plants and algae (Lewis *et al.,* 1989; Jaenicke *et al.*, 1996). Land plants are generally intolerant to large fluctuations in salinity, but are capable of adaptation over time (Atwell *et al.*, 1999). Ammonium uptake is more affected by salinity than $NO_3^-$ uptake (Lewis *et al.,*

1989; Khan *et al.*, 1995; Jaenicke *et al.*, 1996). Ammonium uptake may be more affected by salinity in land plants due simply to $NH_4^+$ assimilation taking place largely in the roots, which are in direct contact with NaCl, as opposed to the leaves, where $NO_3^-$ is assimilated (Lewis *et al.*, 1989).

Nitrate uptake may also counteract the effects of salinity on higher plants to some extent (Lewis *et al.*, 1989; Cordovilla *et al.*, 1995; Khan *et al.*, 1995; Jaenicke *et al.*, 1996). Ammonium does not appear to alleviate sensitivity to NaCl (Cordovilla *et al.*, 1995; Khan *et al.*, 1995). $NO_3^-$ and $Cl^-$ may compete for the same transport proteins, reducing the total uptake of $Cl^-$ (Lewis *et al.*, 1989; Jaenicke *et al.*, 1996). In some plants, $NO_3^-$ and $Cl^-$ use different carriers to transport them through the membrane, but the anions may compete at the tonoplast or at the site of release to the xylem vessels, again leading to a reduction of $Cl^-$ uptake in nitrate-supplied plants (Jaenicke *et al.*, 1996).

Algal species are commonly exposed to large diurnal and seasonal fluctuations in salinity and are generally good osmoregulators (Atwell *et al.*, 1999; Borowitzka, 1999). Marine and estuarine algae such as *M. umbilicatum* usually maintain turgor in varying salinity by an adjustment in internal osmotic potential based on accumulating organic solutes in the cytoplasm (Borowitzka, 1999). Higher plants may accumulate $Ca^+$, $Na^+$ and $K^+$ to act as osmoregulatory ions (Cordovilla *et al.*, 1995).

It is unknown how changes in salinity affect the uptake of nitrogen compounds in euryhaline algae such as *M. umbilicatum*. However, the N uptake mechanisms of these algae are presumably quite robust with regards to salinity as many estuarine species undergo large fluctuations in NaCl concentration on a daily basis. Results of experiments on the effect of salinity on the $\delta^{15}N$ of *M. umbilicatum* are presented in Section 3.7.

### *2.4.7 Other influences*

A number of other factors can influence the uptake, fractionation and storage of N compounds in plants. These include the time of the day and season, nitrogen fixation, uptake of gaseous ammonia, water availability and any associations with mycorrhizal fungi.

Diurnal fluctuations in the amount and $\delta^{15}N$ of nitrogen compounds in the leaves and roots of plants are common. As a result, leaves sampled in the late afternoon may contain up to 20% more nitrogen than leaves sampled at dawn (Hocking *et al.*, 1978; Pate, 1980). It is therefore important to collect plant samples at approximately the same time of the day to avoid this source of error. Also, both herbaceous and woody perennials show marked seasonal differences in their use of N sources and stores (Thornton *et al.*, 1995; Handley and Scrimgeour, 1997). Drawing upon N stores may mean that new growth reflects the N profile of the soil of months before and not its present $\delta^{15}N$. In addition, storage and transport of N compounds often in itself results in fractionation (Yoneyama *et al.*, 1998).

The ability to obtain nitrogen from the atmosphere (via a symbiotic association with specialist bacteria) can also influence the final $\delta^{15}N$ values of some plants. Nitrogen fixation can be described by the reaction:

$$N_2 + 3H_2O \xrightarrow{\textit{nitrogenase}} 2\,NH_3 + 3/2\,O_2 \qquad (5)$$

The large amount of energy required to break the molecular N bond makes the process very inefficient with little associated N-isotope fractionation (Hoefs, 1997). Fixed N therefore has an isotope ratio very similar to that of its source, atmospheric N, and therefore a $\delta^{15}N$ close to zero. Nitrogen-fixing plants therefore make poor "tracer" organisms as their overall $\delta^{15}N$ will be lowered by the influx of fixed N at $\delta^{15}N = 0$.

Gaseous ammonia may also be absorbed by terrestrial and emergent plant leaves via the stomata (Mengel and Kirkby, 1982). The amount of net uptake by this mechanism depends largely on the net partial pressure of $NH_3$ in the atmosphere (Farquar *et al.*, 1980).

The availability of water to terrestrial plants may also influence the final $\delta^{15}N$ signal of their leaves. A study by Handley *et al.* (1994), looked at a number of plant species from sites with different kinds and amounts of water supply. It was found to be impossible to reliably distinguish even between nitrogen-fixing and non-fixing species from different sites on the basis of leaf $\delta^{15}N$. These results suggest that water availability may strongly influence the $\delta^{15}N$ signature of plants as well as their heterogeneity at any one site.

Finally, association with mycorrhizal fungi can affect N cycling in soil and plants and hence fractionation (Handley and Scrimgeour, 1997). These fungi can assimilate large amounts of N, transport N from soil to plant, break down organic N, enhance $N_2$ fixation in N-fixing plants and transport $NH_4^+$ from soil to plant (Handley and Scrimgeour, 1997; Azcon-G-Aguilar *et al.*, 1998). In controlled experiments, plants with mycorrhizal fungal infections were found to vary in $\delta^{15}N$ from uninfected plants of the same species by around 2‰ (Handley *et al.*, 1993; Pate *et al.*, 1993; Azcon-G-Aguilar *et al.*, 1998).

## 2.5 A bioindicator for identifying the major sources of nitrogen in the Hawkesbury-Nepean River and estuary

Once all other confounding factors are removed or allowed for, $\delta^{15}N$ values are effective tracers of nitrogen sources. If the appropriate ancillary data are collected and fractionations between source and sink are accounted for, $\delta^{15}N$ values can provide a powerful tool for examining the sources and sinks of nitrogen compounds in a variety of ecosystems.

Other studies have used marine plants to measure the uptake of sewage-derived N into an aquatic ecosystem, most notable in Australia Costanzo *et al.*, 2001. This study uses marine plants to identify bioavailable sewage N in the Moreton Bay area of Queensland, Australia, but has not attempted to eliminate sources of variability potentially caused by environmental conditions, storage of N by the plants, fractionation, variation between plant species or between individuals. Using a well defined "bioindicator" plant species to collect $\delta^{15}N$ data removes many of the confounding factors commonly associated with nitrogen isotope "tracer" studies. Error associated with trophic level or species differences (Sections 2.3.1 and 2.3.2) can be removed by analysing the $\delta^{15}N$ of only a single, preferably genetically similar, species. Using a laboratory-grown "bioindicator" plant left in the field to take up N for a set period of time also eliminates error associated with turnover or storage of N compounds (Sections 2.4.3 and 3.3). A well-defined "bioindicator" also allows the effect of environmental conditions (Section 3.4.2) to be investigated and quantified, and provides a mechanism to link laboratory and field data.

*Microdictyon umbilicatum* was selected from a list of potential indicator species for use in the Hawkesbury-Nepean River. A number of requirements must be met in order for a plant species to be used to collect valid $\delta^{15}N$ data and thus be a useful bioindicator. These requirements include:

1. Fast turnover of N compounds: to prevent the $\delta^{15}N$ of stored nitrogen compounds from contributing to the final $\delta^{15}N$ of the plant.
2. Fast growth rate, easily cultivated: to reduce the time needed to produce sufficient biomass for experiments and losses due to transplant shock.
3. Genetic similarity: to reduce error due to genetic variation and associated biochemical differences.
4. Endemic to the area they are to be deployed in: to negate problems with introducing noxious or non-native plants to an ecosystem.
5. Free-floating: to restrict nitrogen source to the water column and reduce the influence of sediment nitrogen (rooted plants) and gaseous nitrogen (emergent plants).
6. Non-N-fixing: to eliminate the influence of atmospheric N and symbionts.
7. Structurally simple: to minimise partitioning between plant parts.
8. Produce representative samples: which are homogeneous and easy to process.
9. Simple, well-researched biochemistry: to aid in the design and interpretation of experiments.
10. Tolerant to a wide range of environmental conditions: such as salinity, temperature and light levels, to make it viable for use over the whole river system.
11. Tolerant to high nutrient levels and pollution: for use in heavily eutrophic and polluted waters.

*Micodictyon umbilicatum* was selected as a "bioindicator" suitable for use in the Hawkesbury-Nepean river system, as the literature indicated that it fulfils the requirements outlined above

(Borowitzka *et al.*, 1982). A series of controlled laboratory experiments were carried out to test the growth rate and N turnover time of *M. umbilicatum* under a range of conditions likely to be encountered in the field. Results from these experiments are presented in Section 3.3.2.

*Microdictyon umbilicatum* is a green alga of the family Cladophoraceae (Zechman *et al*, 1990; NCBI, 2000). Considerable attention has been given to this family of algae, particularly to the genus *Cladophora*, and their basic biochemistry is well understood (e.g. Jackson and Hamdy, 1982; Freeman, 1986; Wiencke and Davenport, 1987; Peckol and Rivers, 1991; Pakker *et al.*, 1994). Members of the Cladophoraceae are not known to form symbioses with N-fixing organisms and are not capable of carrying out this process themselves (Round, 1973; Hanisak, 1983; Atwell *et al.*, 1999). It is therefore assumed that there is no fixed N contributing to the N loads of *M. umbilicatum.*

*Micrdictyon umbilicatum* is endemic to NSW coastal areas, and is commonly found in estuaries, often associated with the seagrass *Posidonia* (Borowitzka *et al.*, 1982). For this study, specimens of *M. umbilicatum* were collected from the Barrenjoey area, near the mouth of the Hawkesbury-Nepean River (Figure 2.8). This area is highly eutrophic and frequently has levels of total N and P well above those recommended in the Australian guidelines (NSW EPA, 1994; ANZECC and ARMCANZ, 2000). Plants endemic to this area, such as *M. umbilicatum*, must be therefore relatively tolerant of eutrophic conditions.

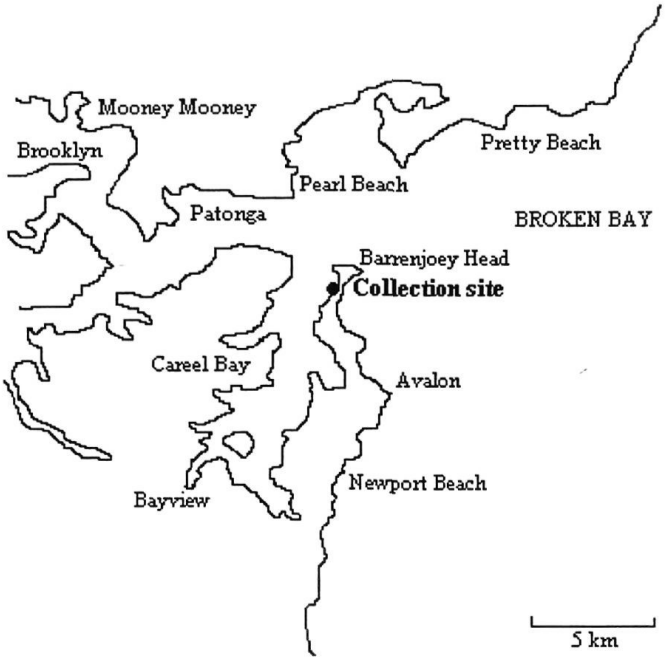



**Figure 2.8**: Map of the collection site of original *Microdictyon umbilicatum* plants used for this study (adapted from WhereiS, 1999).

The thallus of *M. umbilicatum* is comprised of a thin membrane formed from a network of filaments, densely branching in one plane to form a sheet (Hence the Greek roots; *Micro*, small; *dictyon*, net; Figure 2.9). There is little differentiation of the thallus, and hence no possible partitioning of N into separate plant organs (Sections 2.3.3, 2.4.2 and 2.4.3). The delicate, simple structure also meant that samples could be easily processed to homogeneity (for sample processing methods, see Section 3.2.2). Non-homogeneity of processed samples proved a problem in a pilot study where samples of the strap-like, fibrous aquatic ribbon-weed *Vallisneria sp.* were used.

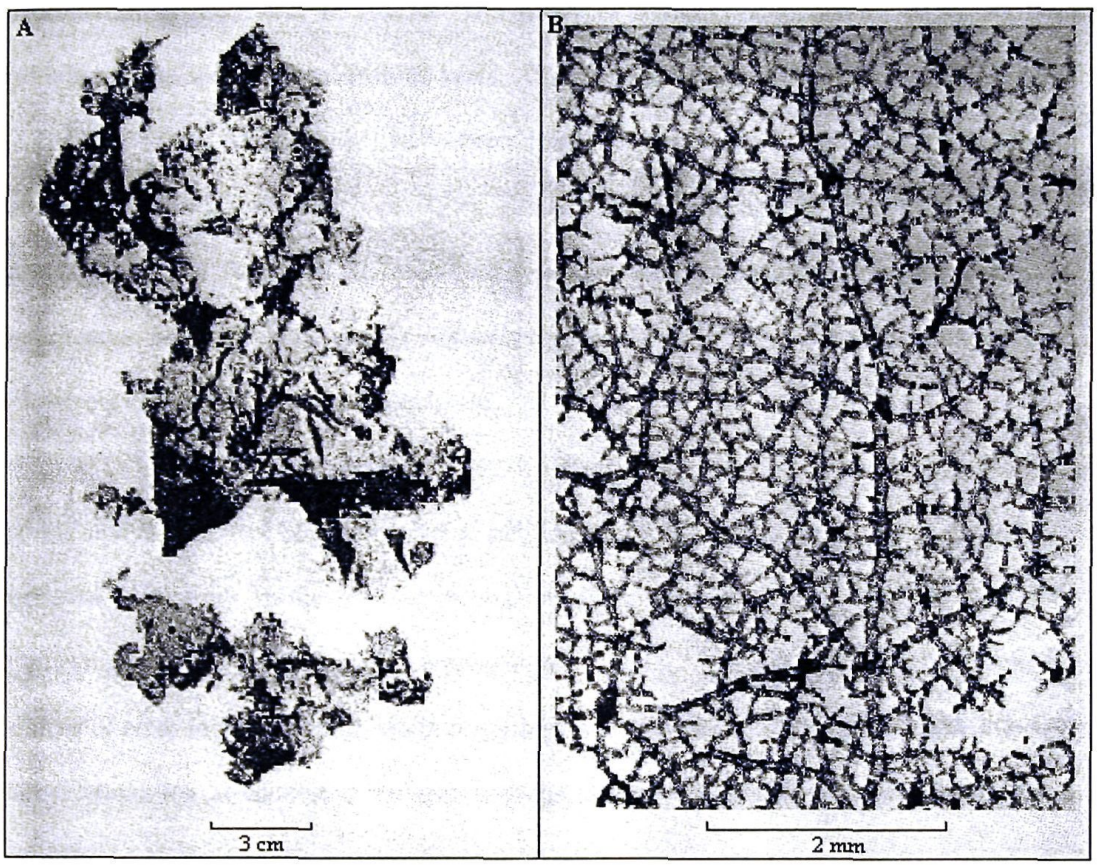



**Figure 2.9:** *Microdictyon umbilcatum;* A. Thallus; B. Thallus detail - branching pattern of the net (adapted from Womersley, 1984).

The holdfast of *Microdictyon* species is generally obscure, and it is common to see *M. umbilicatum* plants floating freely in the water column with no visible holdfast present (Borowitzka *et al.*, 1982; Womersley, 1984; pers. ob.). None of the individual *M. umbilicatum*plants generated in the laboratory during the course of this project developed visible holdfasts and circulated freely in the growth tanks (Section 3.2.1).

Sexual reproduction has not been observed in *M. umbilicatum*, either by other workers (Borowitzka *et al.*, 1982; R. Ritchie, University of Sydney, pers. comm) or in my own culturing of the species for over three years. However, other members of the Cladophoraceae (e.g. *Cladophora*, *Anadyomene*) have been found to have sexual reproduction alternating isomorphic generations (Chapman, 1964; Round, 1973; Bold and Wynne, 1985) and it is possible this also occurs in *M. umbilicatum* under certain conditions. In this study, *M. umbilicatum* was propagated purely vegetatively, and all plants used in the experiments were effectively clones of the original specimens.

In this study, the effect of environmental parameters on *M. umbilicatum* was examined in a series of laboratory studies. The results of these laboratory investigations, and their implications for the field studies, are presented in Chapter 3. A number of other common sources of error in tracer studies using organisms were eliminated in the experimental design, and fractionation at each step between sources and sink were estimated. This approach estimates or eliminates all significant confounding factors from the experiment, allowing the $\delta^{15}N$ values of *Microdictyon* to be used as an effective tracer of bioavailable nitrogen compounds in the Hawkesbury-Nepean river system.

# CHAPTER 3: THE EFFECT OF NUTRIENT CONCENTRATION, LIGHT, TEMPERATURE AND SALINITY ON THE $\delta^{15}N$ OF *MICRODICTYON UMBILICATUM*.

## 3.1 Introduction

Stable isotope ratios can be successfully used to trace the source of nutrients if confounding factors can be eliminated or estimated. The issues underlying this methodology have been discussed in the previous Chapter. Using defined biological material such as *Microdictyon umbilicatum* to absorb nitrogenous signals in the form of $\delta^{15}N$ data from the Hawkesbury-Nepean River removes many of the potential sources of error discussed in detail in Chapter 2.

Errors associated with trophic level or differences between individuals (Sections 2.3.1 and 2.3.2), sample selection (Section 2.3.3) and the potential storage of N compounds (Section 2.4.3) are all removed by the use of laboratory-grown bioindicator algae deployed in the field. All *M. umbilicatum* algae used for the experiments are identical, and hence should all fractionate N in the same way in the absence of different environmental conditions. Experimental error due to the uncertainties of N storage is also eliminated by using *M. umbilicatum* algae grown in the laboratory on a known N source.

### *3.1.1 The turnover of nitrogen compounds in Microdictyon umbilicatum*

The rate of exchange between an organism and its environment can significantly affect its final $\delta^{15}N$ signal. Stored N compounds reflect the nitrogen environment of the organism at the time the compound was formed, and dilute any N uptake from the current environment. The final $\delta^{15}N$ of a plant or alga therefore reflects the balance of the $\delta^{15}N$ of the remaining and newly formed nitrogen compounds .

The rate of N turnover in *M. umbilicatum* under various environmental conditions is a significant influence on the final $\delta^{15}N$ of the algae. For the bioindicator to accurately reflect conditions during exposure, ideally, the algae should be left in the field for sufficient time to come to reflect exclusively the N environment of the river. A series of laboratory experiments was carried out to investigate the rate of N turnover in *M. umbilicatum.*

### *3.1.2 The effect of environmental conditions on $\delta^{15}N$ in Microdictyon umbilicatum*

It is also important to estimate any discrimination between N source and sink. The size and direction of any fractionation can be heavily influenced by environmental conditions. The literature revealed four environmental parameters likely to affect the fractionation of N in *M. umbilicatum.* The amount of discrimination that takes place in algae is, in general, positively correlated with the concentration of $NO_3^-$ in the growth medium. The rate of N uptake, and hence discrimination, is inherently linked to illumination levels. Growth rates, enzymes and the uptake of $NH_4^+$ and $NO_3^-$ are all affected by temperature. Salinity may influence the relative uptake of $NH_4^+$ and $NO_3^-$ in plants and algae, and hence affect their $\delta^{15}N$. Each of these parameters is discussed in detail in Chapter 2.

*Nutrient concentration*

Differences in the concentration and ratio of $NH_4^+$, $NO_3^-$ and other N compounds available to the plant or algae may exert significant influence on the fractionation of N between source and plant and on the distribution of N compounds within higher plants (Section 2.4.2). In general, the amount of discrimination that takes place is often positively correlated with the concentration of $NO_3^-$ in the growth medium. At low concentrations, $NO_3^-$ is taken up by an active transport system, which produces little fractionation. Higher concentrations of $NO_3^-$ are assimilated by a discriminatory enzyme system and may produce significant fractionation

Ammonium concentrations in the Hawkesbury-Nepean River are negligible at most sites (NSW EPA, 1994). Nitrate concentrations are highly variable however, and can be very high at some sites. Discrimination is usually positively correlated with the concentration of $NO_3^-$. It was therefore decided to investigate the effect of $NO_3^-$ concentration, with negligible $NH_4^+$ input.

The effect of $NO_3^-$ source concentration on the turnover and final $\delta^{15}N$ of *M. umbilicatum* was investigated in a series of laboratory experiments. The results of these experiments are presented in Section 3.3.1.

*Light levels*

Both the intensity and quality of light may control the uptake of nitrogen in plants by modulating the assimilation or reduction of compounds (Atwell *et al.*, 1999). In algae such as *M. umbilicatum,* enzymes for assimilating N compounds are supplied with reductants by photosynthesis, either directly or indirectly (Section 2.4.4). This means the rate of N uptake is inherently linked to illumination levels.

For aquatic algae, the main changes to light availability will be caused by depth and turbidity of the water column, and this may vary significantly between sites on the Hawkesbury-Nepean River. Laboratory experiments were therefore done to investigate the effect of light on the final $\delta^{15}N$ of *M. umbilicatum.* The results of these experiments are presented in Section 3.3.2.

*Temperature*

Temperature affects plant and algae growth rates, which in turn influence nitrogen turnover, storage and demand. Plant enzymes are also affected by temperature fluctuations, and will generally suffer thermal stress until they eventually adjust key enzyme reactions to a new optimum. The uptake of $NH_4^+$ and $NO_3^-$ is also directly temperature-dependent in some species (Section 2.4.5).

Any changes in nitrogen uptake may in turn influence the fractionation and turnover of nitrogen compounds, and hence the final $\delta^{15}N$ values of the plant. Experiments investigating the effect of temperature on the turnover and final $\delta^{15}N$ of *M. umbilicatum* were done in the laboratory. The results of these experiments are presented in Section 3.3.2.

*Salinity*

pH can influence the uptake of $NH_4^+$ and $NO_3^-$ into the plant or alga. Ammonium is taken up best in a neutral medium, and uptake is depressed as pH falls. The converse is true for $NO_3^-$, which is taken up more rapidly at low pH values (Section 2.4.6). The main changes to pH in the Hawkesbury-Nepean River are due to salinity. Salinity may also affect the uptake of other N compounds indirectly via its influence on the relative uptake of $NH_4^+$ and $NO_3^-$. Ammonium uptake is more affected by salinity than $NO_3^-$ uptake (Section 2.4.6).

Estuarine algae, such as *M. umbilicatum*, usually osmoregulate by accumulating organic solutes or ions in the cytoplasm (Section 2.4.6). It is not known how the accumulation of these compounds may affect the overall $\delta^{15}N$ of the algae but any modifications to biochemical pathways in response to salinity fluctuations may lead to differences in the fractionation of nitrogen (see Sections 2.3.1, 2.4.1 and 2.4.6). The effect of salinity on the turnover and final $\delta^{15}N$ of *M. umbilicatum* was investigated in a series of laboratory experiments. The results of these experiments are presented in Section 3.3.2.

### *3.1.3 Aims of the laboratory experiments*

The effect of environmental conditions on the $\delta^{15}N$ of *M. umbilicatum* were investigated and quantified in a series of laboratory experiments. Specifically, the effect of the following environmental parameters were investigated:

1. Concentration of N species.
2. Ambient light levels (Photosynthetically Active Radiation, PAR).
3. Water temperature.
4. Salinity.

The rate of N turnover in *M. umbilicatum* was also investigated under a range of environmental conditions. Finally, the results of these experiments were interpreted in terms of their implications for the field experiments (presented in Chapter 5).

## 3.2 Materials and methods

### *3.2.1 Collection and culture of Microdictyon umbilicatum*

Original *M. umbilicatum* specimens for culturing were collected from Barrenjoey, N.S.W (Figure 2.8). Specimens were collected on SCUBA in 2-6 m of water and were immediately transferred to polyurethane jars containing 500mL of seawater from the collection site. Jars containing algae were transported back to the laboratory in an insulated container. At the laboratory, the algae were rinsed in filtered seawater to remove any visible epiphytes or entangling material. The thallus was cut into pieces approximately 5cm in diameter before being transferred to the growth tanks (Figure 3.1).

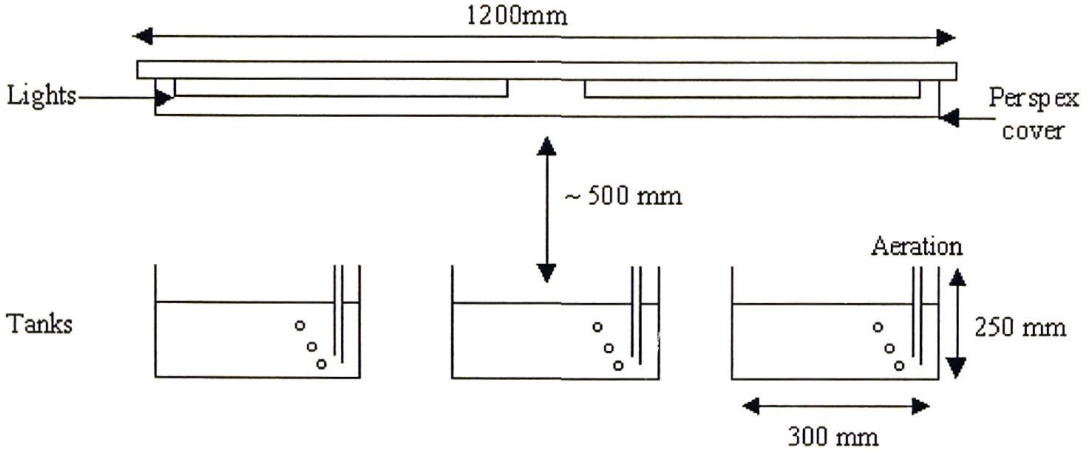



**Figure 3.1**: Arrangement of tanks for the culture of *Microdictyon umbilicatum* in the laboratory.

Tanks were kept in a room with a constant temperature of 25°C. Each tank was aerated and circulated by an air-stone in one corner, powered by a small commercial aquarium pump. Light was provided by a bank of 2 Gro-Lux and 4 Cool White fluorescent tubes, giving a combined illumination of approximately 120 lux at the level of the algae. The lights were attached to an automatic timer, giving 14 hours of illumination and 10 hours of darkness per day. All algae were initially grown in seawater, but were changed to other salinity regimes suitable for their location for field experiments (see Section 5.2.2). Seawater was collected from Sydney Aquarium, Darling Harbour, N.S.W., in polyurethane containers reserved for this purpose and filtered through 0.22 µm glass fibre filters before being stored in sealed polyurethane containers before use.

Algae were supplied with N as potassium nitrate at a concentration of $0.05 mg.L^{-1}$. Half the algae were grown in a nutrient solution with a $\delta^{15}N$ of 10‰, and half were grown in a solution with a $\delta^{15}N$ of 5‰. These values were selected as they were easily commercially available as nutrient solutions. These algae provided the initial "high" and "low" $\delta^{15}N$ algae used in the turnover experiments (Section 3.2.3) and as the N turnover controls used in the field experiments (Chapter 5). Later, it was found that field $\delta^{15}N$ values could be as high as 17‰, but all algae (both initial "high" and initial "low" algae) attained the same final value. The growth medium was completely renewed once per week (except where noted) during the course of the experiments, to maintain the nutrient concentrations at a relatively constant level.

Algae were divided in two approximately once a week to encourage continued growth of the thallus and to provide a large number of suitably-sized algae for experiments. All algae used in both the laboratory and field experiments described in this thesis were approximately 7cm in diameter.

### *3.2.2 General experimental conditions*

*Microdictyon umbilicatum* algae were cultured under normal conditions for all experiments discussed in this Chapter (Section 3.2.1). Half the algae for each experiment were from the "high $\delta^{15}N$" culture group ($\delta^{15}N$ = 10‰; Section 3.2.1) and half were from the "low $\delta^{15}N$" culture group ($\delta^{15}N$ = 5‰; Section 3.2.1). Four algae were randomly selected from each group to provide an initial measure for each culture group ("initial" or "Day 0" samples where presented). The remainder of the algae required were switched to a tank with the opposite $\delta^{15}N$ to their culture conditions at the start of the experiment. This provided a control for N turnover for each experiment. Apart from the factor under investigation (nutrient $\delta^{15}N$, concentration, light level, salinity or temperature), conditions in the tanks were identical to those in the culture tanks (Section 3.2.1).

### *3.2.3 The turnover of nitrogen compounds in Microdictyon umbilicatum*

Eighty algae were cultured, half in "high $\delta^{15}N$" conditions and half in "low $\delta^{15}N$" conditions, as usual (Section 3.2.2). Four algae from each group were sampled at the beginning of the experiment to provide "initial" $\delta^{15}N$ values for the group. The remainder of the algae were swapped into the opposite $\delta^{15}N$ nutrient conditions from those they were cultured in i.e. algae from the "high $\delta^{15}N$" group were transferred to "low $\delta^{15}N$" conditions and vice versa. Analysing samples for $\delta^{15}N$ would then provide a measure of the proportion of stored to new N compounds present in the algae at any given time.

Four algae were randomly sampled from each experimental treatment on days 1, 3, 5, 7, 10, 14, 17 and 21 of the experiment. Nutrient solutions in each tank were renewed every 2 days to ensure a relatively consistent $\delta^{15}N$ environment. Samples were prepared and analysed in

the usual manner (Section 3.2.5). This experiment was replicated twice, once in June 1997, and once in March 1999.

### *3.2.4 The effect of environmental conditions on the $\delta^{15}N$ of Microdictyon umbilicatum*

Algae were cultured, half in "high $\delta^{15}N$" conditions and half in "low $\delta^{15}N$" conditions, as usual (Section 3.2.2). A turnover control was provided in each experiment by initially sampling four algae from each group, then switching the remaining algae to the opposite $\delta^{15}N$ nutrient regime to that in which they were cultured in (Figure 3.2). These turnover results are not presented here, but were considered acceptable for these experiments if algae had reached the $\delta^{15}N$ of their new nutrient regime (allowing for fractionation) within 10 days.

Algae from each nutrient regime were then randomly allocated to a treatment in each of the experiments (Figure 3.2). Culture solutions were renewed every 2 days for the duration of the experiments to ensure that nutrient conditions remained approximately constant.

Algae were grown in these new conditions for 3 weeks, with 4 from each group randomly selected for analysis on days 7, 14 and 21 (Figure 3.2). Each experiment investigating the effect of environmental parameters on the $\delta^{15}N$ of *M. umbilicatum* were replicated twice, once in August 1998 and once in March 1999.

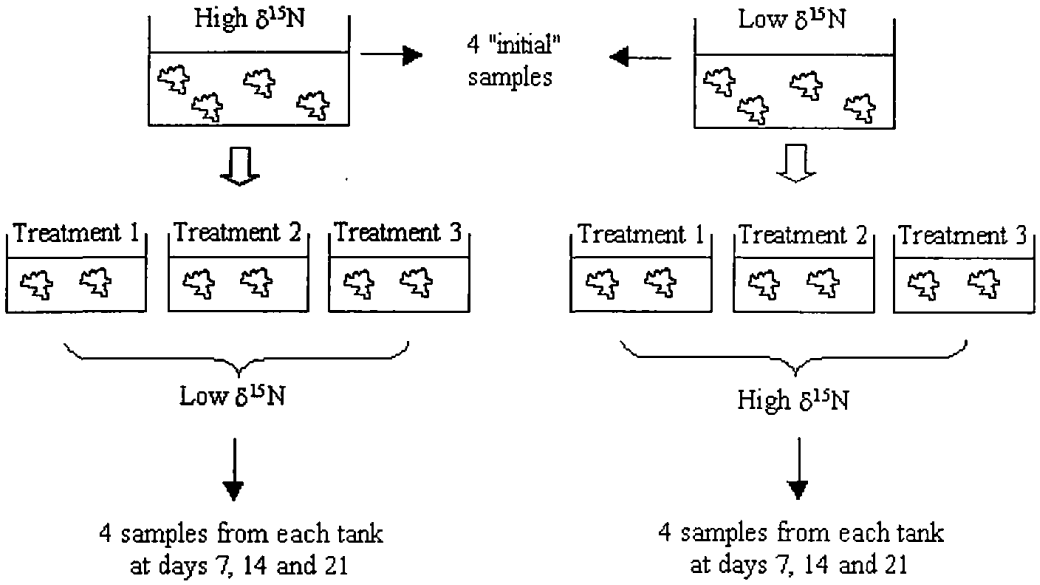



**Figure 3.2**: General design of experiments investigating the effect of environmental conditions on the $\delta^{15}N$ of *Microdictyon umbilictatum*. Initially, there were three growth tanks at each nutrient regime. Eventually, up to ten growth tanks at each regime were operating.

*Nutrient concentration*

Algae were randomly allocated to one of three treatments:

1. High $NO_3^-$ concentration (N concentration = 5 $mg.L^{-1}$).
2. Medium $NO_3^-$ concentration (N concentration = 0.5 $mg.L^{-1}$).
3. Low $NO_3^-$ concentration (N concentration < 0.1 $mg.L^{-1}$).

These concentrations were chosen as likely to produce a change in fractionation due to concentration as seen by other researchers (Evans *et al.*, 1996) and were also representative of the range of nitrate concentrations in the Hawkesbury-Nepean River (NSW EPA, 1994). Nutrient concentrations were prepared as potassium nitrate in solution, and solutions were renewed every 2 days for the duration of the experiment to ensure that concentrations remained approximately constant.

*Light levels*

For this experiment, light levels (PAR) were altered in the tanks using commercial shade-cloth, which was layered until light levels were half and one third of the available light, measured using a light meter. Algae were randomly allocated to one of 3 treatments:

1. Full (full available light = approximately 120 lux).
2. Half (half available light = approximately 60 lux).
3. Third (one third available light = approximately 40 lux).

*Temperature*

Algae were randomly allocated to one of 5 temperature treatments:

1. 10°C.
2. 15°C.
3. 20°C.
4. 25°C.
5. 30°C.

Temperature was controlled by placing the tanks in temperature-controlled rooms or by the use of commercial aquarium heaters in the tanks.

*Salinity*

Algae were randomly allocated to one of five salinity treatments:

1. 0 ppt.
2. 10 ppt.
3. 20 ppt.
4. 30 ppt.
5. 40 ppt.

These salinities were selected as representative of the range of salinities between field sites in the Hawkesbury-Nepean River (NSW EPA, 1994). Different salinities were achieved by the concentration and dilution of seawater from the usual source (Section 3.2.2). Seawater was concentrated by placing it in 500mL glass beakers in a 60°C oven for three days, producing a super-saturated seawater solution which was then used to produce each of the saline solutions used in the experiments. Concentrated seawater was diluted with distilled water until the correct reading was obtained on a salinity meter.

### *3.2.5 Sample preparation and analysis*

All samples from the laboratory and field experiments, once in the laboratory, were immediately washed under running tap water, then rinsed with distilled water. Any visible epiphytes were removed, along with any areas that appeared discoloured or unhealthy. Samples were then frozen at -20°C for storage or immediately processed. Samples were oven rather than freeze-dried because of the large numbers involved. Samples were arranged in glass Petri dishes and were placed in an oven at 60°C until they were completely dry (3 days). Each sample was then ground to a fine powder using a laboratory mill grinder and stored in airtight Eppendorf tubes at room temperature until analysed. Samples were analysed for their $\delta^{15}N$ using a mass spectrometer, an instrument that separates charged atoms on the basis of their mass differences.

Samples for this project were analysed by Steven Howe at the Stable Isotope Ratio Mass Spectrometry Lab, The University at Albany, New York, USA. Analysis was done using a Carlo Erba NA 1500 Series II NC elemental analyser (for details see Stable Isotope Ratio Mass Spectrometry Lab, 1999). Internal working standards of:

1. IAEA-N3, a potassium nitrate standard, with a $\delta^{15}N$ of + 4.69
2. USGS-25, an ammonium sulfate standard, with a $\delta^{15}N$ of -30.25
3. USGS-26, an ammonium sulfate standard, with a $\delta^{15}N$ of +53.62

were analysed at regular intervals during the auto-runs (for a discussion of these standards, see Böhlke and Coplen, 1995). The elemental analyser was interfaced to a Micromass Optima gas-source triple-collector mass spectrometer.

## 3.3 Results

Experiments showed that full turnover of N compounds occurs in *M. umbilicatum* algae in less than 14 days (Section 3.3.1). Therefore, in all experiments investigating the effect of environmental condition on the $\delta^{15}N$ *of M. umbilicatum*, results from Day 14 are presented as full equilibration to the new nutrient regime was achieved in this time. (Samples from days 7 and 21 were periodically analysed to ensure turnover was complete under each regime, but these results are not presented ). Algae were deployed in the water for 14 days for the field experiments (Chapter 5). Results are presented below as mean $\delta^{15}N$ fractionation, where:

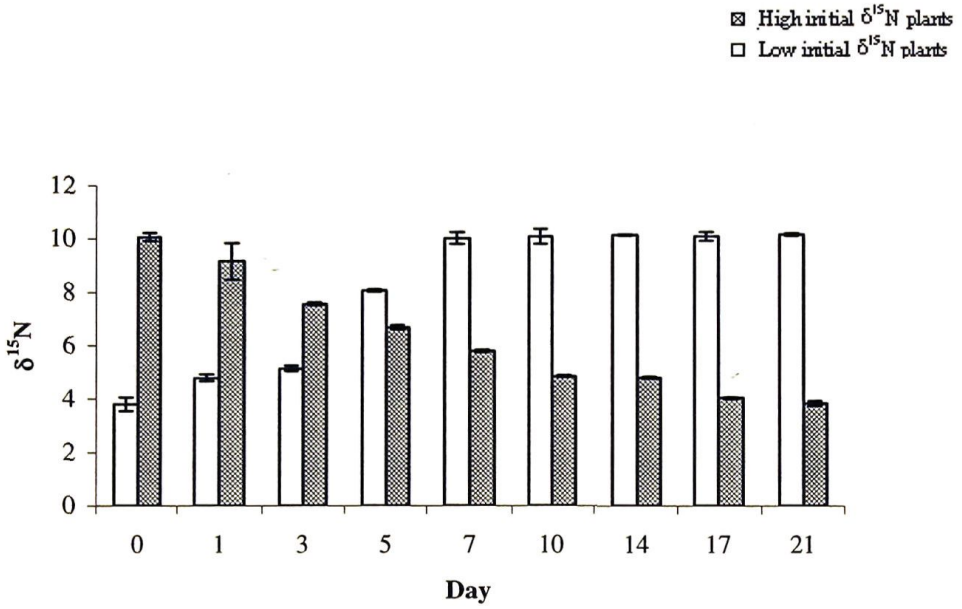
Fractionation = $\delta^{15}N_{source} - \delta^{15}N_{sink}$

### *3.3.1 The turnover of nitrogen compounds in Microdictyon umbilicatum*

Nitrogen turnover in *M. umbilicatum* was very rapid, with the bulk of N compounds exchanged within 7 days. Algae from both "high" ($\delta^{15}N$ = 10‰) and "low" ($\delta^{15}N$ = 5‰) initial $\delta^{15}N$ regimes had equilibrated to the $\delta^{15}N$ of their new nutrient environment by day 7, and maintained this $\delta^{15}N$ for the duration of the experiments (Figure 3.3). Once significant differences were no longer moving downwards, it was deemed that the N compounds in the algae had turned over completely. This pattern was consistent for both replicate experiments (Figure 3.3).

A consistent difference of approximately -1‰ can be seen between the $\delta^{15}N$ of the nutrients provided and the final $\delta^{15}N$ of the *M. umbilicatum* algae themselves (Figure 3.3). This is due to internal fractionation of the N compounds by the algae and was consistent under normal conditions among all laboratory experiments (see Sections 3.3.1 and 3.3.2).

**A**: July 1997.




**B**: March 1999.

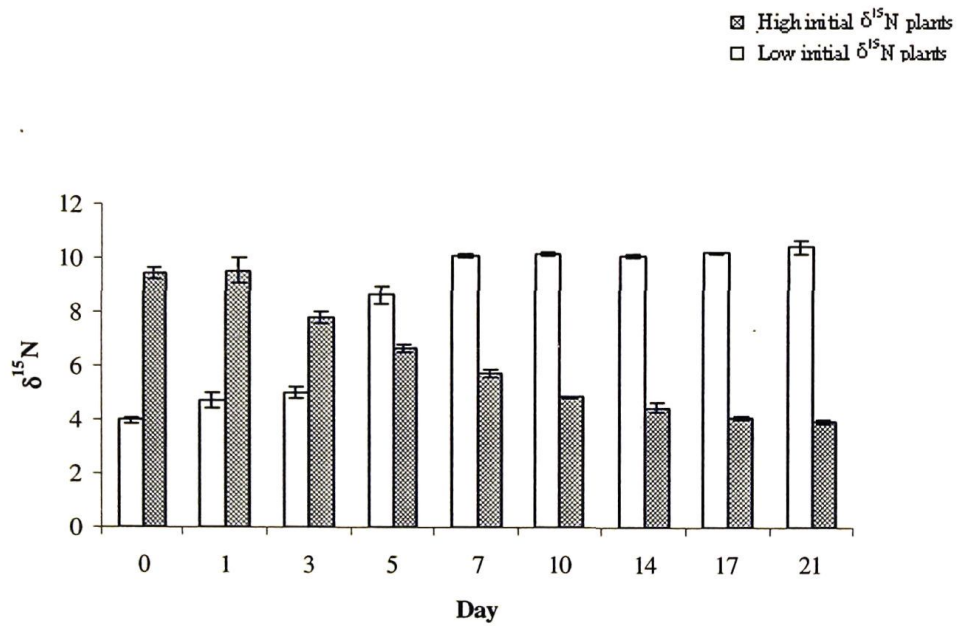



**Figure 3.3**: Rate of nitrogen turnover in *Microdictyon umbilicatum* in 2 replicate experiments (values are mean $\delta^{15}N \pm$ SD (‰) from the 3 replicate algae sampled on each day).

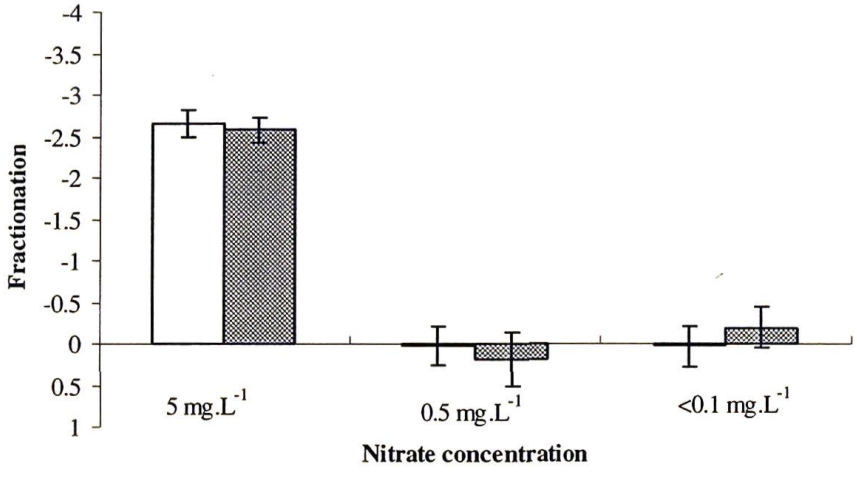
### *3.3.2 The effect of environmental conditions on the $\delta^{15}N$ of Microdictyon umbilicatum*

*Nutrient concentration*

Nutrient concentration in the growth media exerted considerable influence on fractionation in *M. umbilicatum*. Algae grown in a high nitrate concentration (5mg.$L^{-1}$) had a $\delta^{15}N$ lower than the growth media by a mean of -2.7 ± 0.2‰ (Figure 3.4).

Algae grown in a nutrient concentration of less than 0.5 mg.$L^{-1}$ nitrate (the "medium" and "low" treatments; Section 3.2.4) were unaffected however, giving a mean fractionation of only approximately -1‰, consistent with the fractionation of N by *M. umbilicatum* under normal growth conditions (see Section 3.3.1). This pattern seen in the first replicate experiment was repeated in the second experimental trial (Figure 3.4).

**A**: August 1998.




**B**: March 1999.

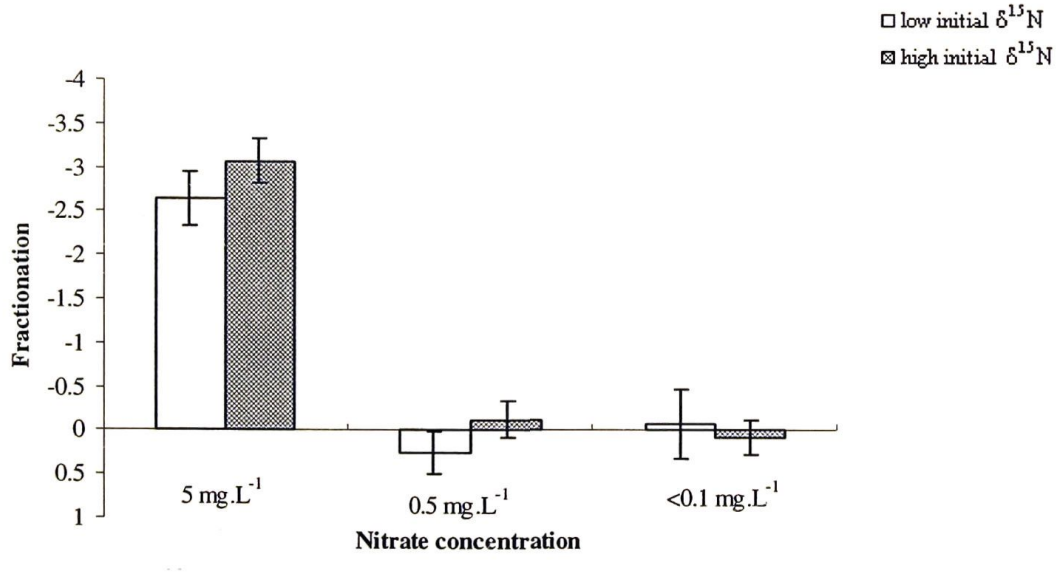


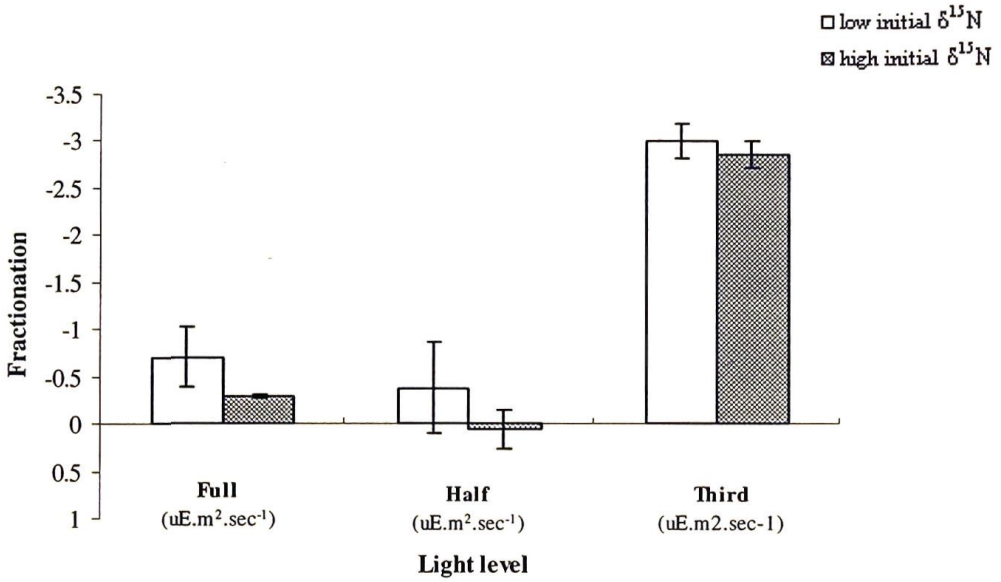

**Figure 3.4**: The effect of nitrate concentration on $\delta^{15}N$ in *Microdictyon umbilicatum* at 14 days (values are mean $\delta^{15}N$ fractionation ± SD (‰) from the 4 replicate algae sampled).

*Light levels*

Fractionation in *M. umbilicatum* was substantially influenced by light levels. s grown in one third of available light had a $\delta^{15}N$ lower than the growth media by a mean of -3.0 ± 0.1‰ (Figure 3.5).

Algae grown in light levels of half or full available light were unaffected however, giving a mean fractionation of only approximately -1, consistent with the fractionation of N by *M. umbilicatum* under normal growth conditions (see Section 3.3.1). This pattern is consistent for both replicate experiments (Figure 3.5).

**A**: August 1998.




**B**: March 1999.

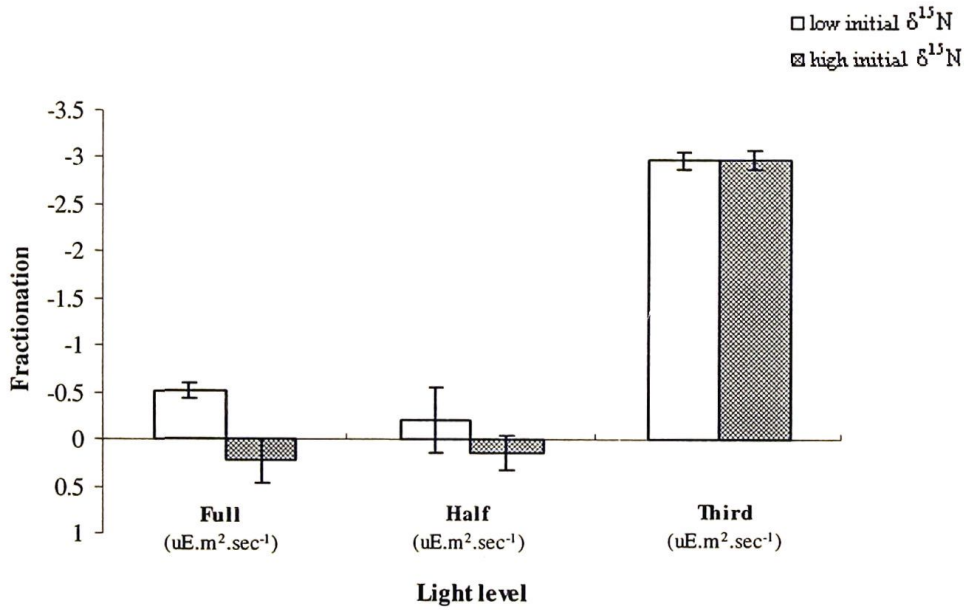


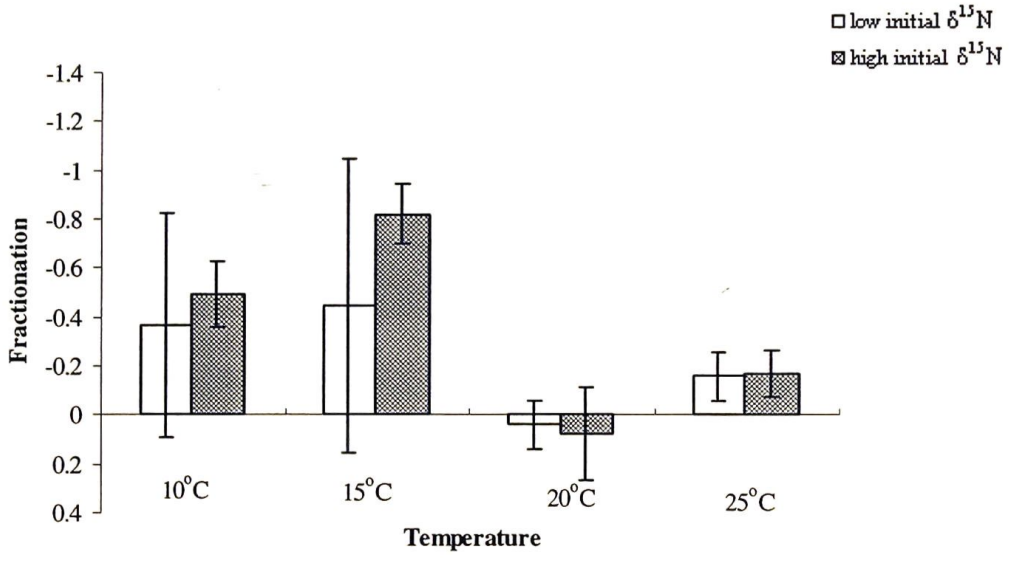

**Figure 3.5**: The effect of light levels on $\delta^{15}N$ in *Microdictyon umbilicatum* at 14 days (values are mean $\delta^{15}N$ fractionation ± SD (‰) from the 4 replicate algae sampled).

*Temperature*

Fractionation in *M. umbilicatum* was not strongly influenced by temperature (Figure 3.6). Algae grown at all temperatures gave a mean fractionation of only approximately -1, consistent with the fractionation of N by *M. umbilicatum* under normal growth conditions (see Section 3.3.1). Algae would not grow properly at 30°C, so no results are presented for this temperature regime.

There is some variation between different temperature treatments and replicate experiments, but no consistent pattern is evident once error is taken into account (Figure 3.6). It should also be noted that the scale on which any variation due to temperature is seen is small (range = approximately 1.1‰; Figure 3.6).

**A**: August 1998.




**B**: March 1999.

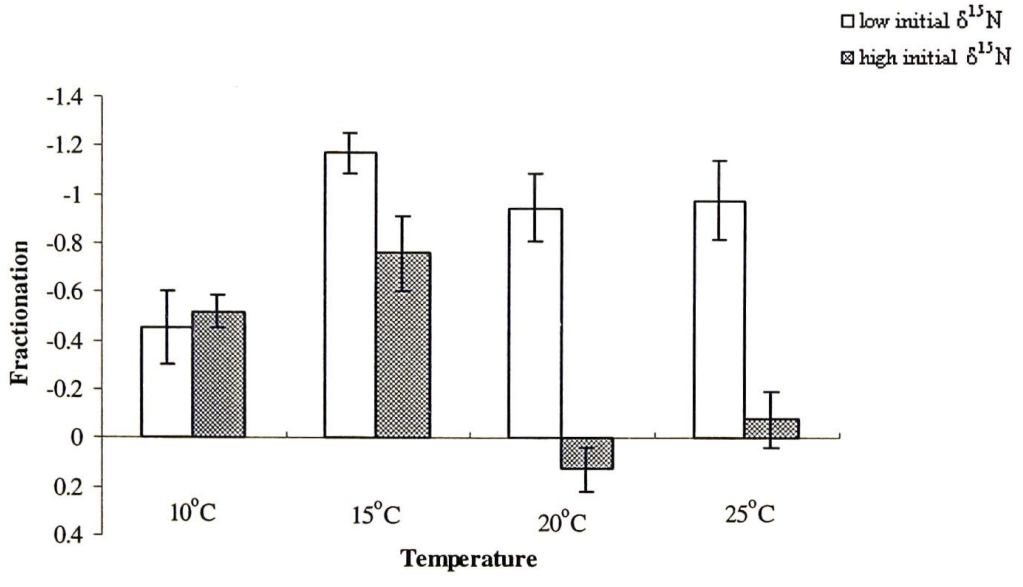


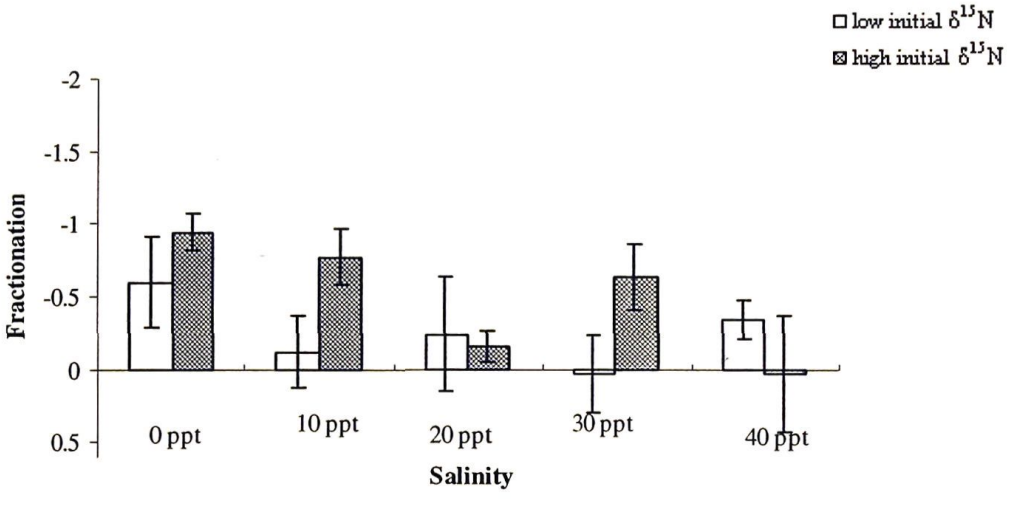

**Figure 3.6**: The effect of temperature on $\delta^{15}N$ in *Microdictyon umbilicatum* at 14 days (values are mean $\delta^{15}N$ fractionation ± SD (‰) from the 4 replicate algae sampled).

*Salinity*

Fractionation in *M. umbilicatum* was not strongly influenced by salinity (Figure 3.7). Algae grown at all salinities gave a mean fractionation of only approximately -1, consistent with the fractionation of N by *M. umbilicatum* under normal growth conditions (see Section 3.3.1).

There is some variation between different salinity treatments and replicate experiments, but no consistent pattern is evident once error is taken into account (Figure 3.7). It should also be noted that the scale on which any variation due to temperature is seen is small (range = approximately 1.5‰; Figure 3.7).

**A**: August 1998.




**B**: March 1999.

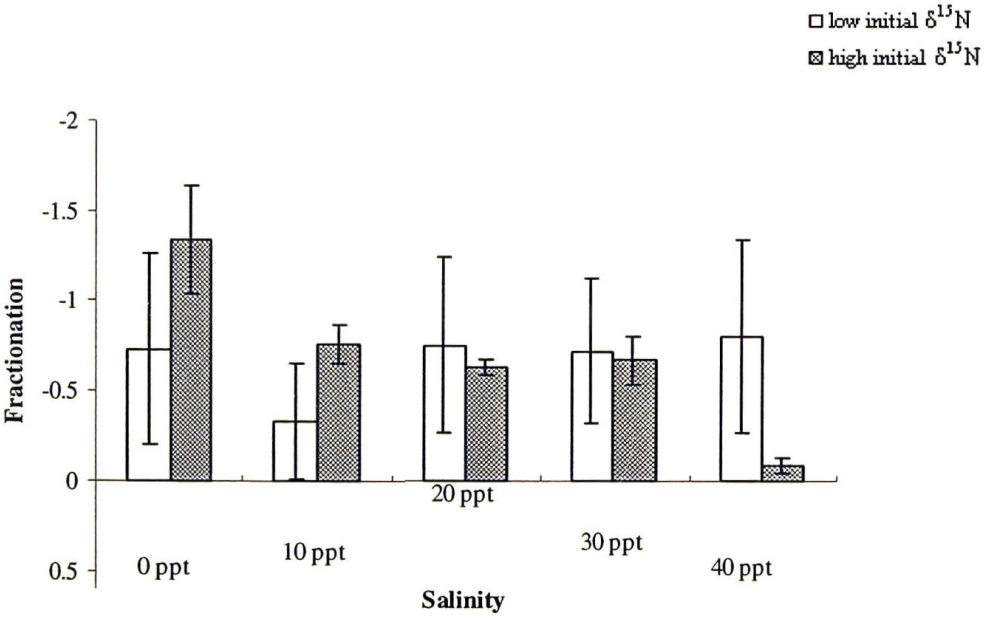



**Figure 3.7**: The effect of salinity on $\delta^{15}N$ in *Microdictyon umbilicatum* at 14 days (values are mean $\delta^{15}N$ fractionation ± SD (‰) from the 4 replicate algae sampled).

## 3.4 Discussion

### *3.4.1 The turnover of nitrogen compounds in Microdictyon umbilicatum*

Nitrogen turnover in *M. umbilicatum* was very rapid. A stored N pool may make up a large portion of the total nitrogen content of algae, significantly altering their stable isotope ratio. In *M. umbilicatum* however, stored N compounds had been totally replaced within the algae within 10 days (Figure 3.3), leaving only compounds formed with a $^{15}N$ ratio reflecting that of the new medium.

This rapid turnover makes *M. umbilicatum* ideal for a tracer study such as the one undertaken in this project. As the algae quickly come into equilibrium with their environment, they will be an accurate reflection of the $\delta^{15}N$ of any nitrogen compounds available. Under ideal laboratory conditions, N was turned over in 7-8 days, but may be slightly slower under field conditions. For this reason, it was decided to leave the algae in the field for two weeks, to allow them to equilibrate fully with their N environment under possibly variable conditions.

### *3.4.2 The effect of environmental conditions on $\delta^{15}N$ in Microdictyon umbilicatum*

*Nutrient concentration*

The concentration of nitrate in the growth medium had considerable influence on the fractionation of N in *M. umbilicatum*, with high concentrations of nitrate producing much greater fractionations than low nitrate concentrations (Figure 3.4). The level of discrimination by *M. umbilicatum* at high $NO_3^-$ concentrations (-2.7 ± 0.2‰, Figure 3.4) is within the wide range of 0 to –24‰ reported for other plant and algae species grown in millimolar concentrations of $NO_3^-$ (Table 2.1; Hoch *et al.*, 1992; Fogel and Cifuentes, 1993; Evans *et al.*, 1996). However, the discrimination of approximately -1% by *M. umbilicatum* at concentrations of $NO_3^-$ < 0.5 mg.$L^{-1}$ is smaller than the -4 to -10‰ reported in the literature (Table 2.1).

Algae such as *M. umbilicatum* tend to take up $NH_4^+$ preferentially, often to the extent of switching to $NO_3^-$ uptake only when $NH_4^+$ concentrations in the medium are negligible (Morris, 1974; Raven *et al.*, 1992). Ammonium concentrations at the field sites selected in the Hawkesbury-Nepean River are very low (mean for all sites = 0.059 mg.$L^{-1}$; NSW EPA, 1994), however, this does not indicate the availabilty of $NH_4^+$for plant and algal growth. Differing concentrations of available ammonium have been found in other studies to affect the partitioning of N to different plant organs (Yoneyama and Kumazawa, 1972; Yoneyama and Ishizuka, 1982; Yoneyama and Kaneko, 1988). If the primary source of N for *M. umbilicatum* in the field is $NO_3^-$, this would remove the possibility of any partitioning of N to different plant organs due to N source. However, even in the presence of significant levels of available $NH_4^+$, *M. umbilicatum* is relatively undifferentiated and has a very rapid turnover of N. It is therefore unlikely that any type of partitioning between organs would occur.

Discrimination can also occur when $NO_3^-$ is the sole N source, with the level of discrimination usually positively correlated with the concentration of $NO_3^-$ in the growth media (Table 2.1; Kohl and Shearer, 1980; Mariotti *et al.*, 1981; Evans *et al.*, 1996). The level of discrimination between N source and *M. umbilicatum* is also strongly correlated with $NO_3^-$ concentration (Figure 3.4).

Concentrations of $NO_3^-$ of 5 $mg.L^{-1}$ may be high enough to effectively mimic an open system in *M. umbilicatum*, supplying the substrate at a greater rate than can be taken up by the assimilatory enzymes and therefore allowing them to preferentially take up the lighter isotope (see Section 2.2.3). Lower $NO_3^-$ concentrations, which would mimic a closed system, may be taken up in *M. umbilicatum* by an active transport system, which is normally non-discriminatory (Evans *et al.*, 1996).

At some concentration of $NO_3^-$ between 0.5 and 5 $mg.L^{-1}$, the lighter isotope is able to begin to be taken up preferentially by *M. umbilicatum*, lowering the final $\delta^{15}N$ of the alga. The exact concentration at which this effect begins, and whether the effect is total at some point of gradual over a range of concentrations is unknown. For the purpose of this work, concentrations of $NO_3^- \leq 5$ $mg.L^{-1}$ were adjusted, and concentrations lower than this were left unadjusted. Concentrations of $NO_3^-$ greater than 5 $mg.L^{-1}$ have been recorded at a number of the sites (NSW EPA, 1994) in the Hawkesbury-Nepean River where *M. umbilicatum* algae were deployed as a $\delta^{15}N$ "bioindicator" (Figure 5.4). Corrections to the final $\delta^{15}N$ values of *M. umbilicatum*, to allow for the enhanced fractionation caused by high $NO_3^-$ concentrations, is will therefore be necessary at these sites.

*Light levels*

Light levels also have a considerable influence on the fractionation of N in *M. umbilicatum*. When light levels were cut down to one third of the available light (ie > 40 lux; Section 3.2.4), discrimination increased markedly from normal levels of approximately -1‰ to a mean of -3.0 ± 0.1‰.

Nitrate reductase in algae such as *M. umbilicatum* is supplied with electrons by NADPH. This directly links light levels with the rate of $NO_3^-$ assimilation in algae via the rate of photosynthetic production of NADPH. The rate of $NO_3^-$ uptake was not directly measured in these experiments, but it can be assumed that it was depressed in *M. umbilicatum* algae grown under low light conditions compared to algae grown in full light conditions.

If the rate of $NO_3^-$ uptake in *M. umbilicatum* is depressed via the NADPH supply under low light conditions, the enzyme assimilatory step may become the rate-limiting one (Section 2.2.2; Figure 2.2). This effectively allows any discrimination associated with this step to be fully expressed (Shearer and Kohl, 1993). Nitrate reductase may have a fractionation of up to –15‰ associated with it (Wada and Hattori, 1991; Yoneyama *et al.*, 1998).

Fractionation by nitrate reductase may account for the increased discrimination between N source and *M. umbilicatum* under low light conditions. Under high light intensities, this discrimination is unlikely, as the enzymatic assimilation step is supplied with adequate photosynthetic reductants and is no longer rate-limiting (Shearer and Kohl, 1989). *Microdictyon umbilicatum* discriminated only nominally against $^{15}N$ under full light conditions (Figure 3.5).

Ammonium assimilation may also be linked to light intensity (Ullrich, 1987; Atwell *et al.*, 1999). Low levels of illumination may produce fractionation of $NH_4^+$ via similar mechanisms to $NO_3^-$ (see above).

Turbidity levels are quite high at a number of the sites (NSW EPA, 1994) in the Hawkesbury-Nepean River where *M. umbilicatum* plants were deployed as a $\delta^{15}N$ "bioindicator" (Figure 5.4). This turbidity may reduce light intensity at these sites to a level low enough to produce substantial discrimination in *M. umbilicatum*. Corrections to the final $\delta^{15}N$ values of *M. umbilicatum*, to allow for the enhanced fractionation caused by low light levels, is therefore necessary at these sites.

*Temperature*

Temperature had no discernible influence on the fractionation of N in *M. umbilicatum*. However, temperatures above 30°C dramatically reduced growth, and algae from this treatment were not fit to analyse in either of the experiments. This is unlikely to affect the field experiments however, as temperatures of > 21°C have not been recorded in the Hawkesbury-Nepean River (NSW EPA, 1994).

The uptake of $NH_4^+$ and $NO_3^-$ is temperature-dependent in some plant and alga species (Mengel and Kirkby, 1982). However, the manner in which uptake is affected differs between species. The rate of uptake of both ions is generally depressed at lower temperatures (Clarkeson and Warner, 1979; Mengel and Kirkby, 1982). The rate of $NH_4^+$ and $NO_3^-$ uptake was not directly measured in these experiments, but depression of N uptake at low temperatures was also likely in *M. umbilicatum*.

Temperature often affects the rate of uptake of $NO_3^-$ more than $NH_4^+$ (Clarkeson and Warner, 1979; Vaast *et al.*, 1998). This may result in significant changes to the final $\delta^{15}N$ of the plant or alga if available $NH_4^+$ and $NO_3^-$ have very different $\delta^{15}N$ values. However, in the Hawkesbury-Nepean River, $NH_4^+$ concentrations are very low (NSW EPA, 1994), and are unlikely to significantly contribute to the overall $\delta^{15}N$ of *M. umbilicatum*. Any relative changes to the uptake of $NH_4^+$ and $NO_3^-$ due to temperature are therefore not relevant to this study.

Temperature fluctuations or differences between sites in the Hawkesbury-Nepean River are also small (NSW EPA, 1994), and are unlikely to be significant over the 2 week duration of the field experiments (Section 5.2.2). Fractionation of N by *M. umbilicatum* at temperatures between 10 and 25°C was negligible in any case (Figure 3.6). It was therefore determined to be unnecessary to adjust the final $\delta^{15}N$ of *M. umbilicatum* to allow for the influence of temperature.

*Salinity*

Salinity also had no discernible influence on the fractionation of N in *M. umbilicatum*. The uptake of $NH_4^+$ and $NO_3^-$ is affected by salinity in many plant and algae species (Khan *et al.*, 1995; Atwell *et al.*, 1999). The rate of $NH_4^+$ and $NO_3^-$ uptake was not directly measured in these experiments, but this is also likely to be the case in *M. umbilicatum*.

In higher plants, $NH_4^+$ uptake is influenced by salinity more then $NO_3^-$ uptake (Khan *et al.*, 1995; Jaenicke *et al.*, 1996). This pattern may also occur in algae, but has not been investigated for many species. Changes to the relative uptake rates of $NH_4^+$ and $NO_3^-$ may result in significant changes to the final $\delta^{15}N$ of the plant or alga if available $NH_4^+$ and $NO_3^-$ have very different $\delta^{15}N$ values. However, in the Hawkesbury-Nepean River, $NH_4^+$

concentrations are very low (NSW EPA, 1994), and are unlikely to significantly contribute to the overall $\delta^{15}N$ of *M. umbilicatum*. Any relative changes to the uptake of $NH_4^+$ and $NO_3^-$ due to salinity are therefore not relevant to this study.

Estuarine algae such as *M. umbilicatum* are often very good osmoregulators, and are capable of growth in a wide variety of salinities (Borowitzka, 1999). *M. umbilicatum* was found to be extremely tolerant to a wide range of salinities and was cultured for field experiments in salinities ranging from freshwater to full seawater (Section 5.2.2). The mechanism by which osmoregulation is achieved in *M. umbilicatum* is unknown, however it is likely to be by the accumulation of organic solutes as is common for algae (Borowitzka, 1999). If this is the case, the production of these compounds in saline conditions does not affect the overall fractionation of N compounds, as there was no relationship between discrimination and salinity seen in *M. umbilicatum*.

Fractionation of N at salinities ranging from 0 to 40 ppt was negligible (Figure 3.7). It was therefore determined to be unnecessary to adjust the final $\delta^{15}N$ of *M. umbilicatum* to allow for the influence of salinity.

The concentration of nutrients and the availability of light to *M. umbilicatum* had a marked effect on the level of fractionation between source and alga $\delta^{15}N$. In general, the biochemistry of the alga was found to discriminate marginally against the heavier isotope, producing a fractionation of approximately -1‰ from source to alga. This level of discrimination was unaffected by temperature or salinity. High nutrient concentration and low light levels however, increased fractionation to up to 3‰ (Section 3.3.2).

## 3.5 Implications for field experiments

The results of the laboratory experiments described in this Chapter were used, if necessary, to adjust the final $\delta^{15}N$ values of algae deployed in the field to allow for the influence of environmental conditions. This allows N source $\delta^{15}N$ to be effectively isolated from other factors contributing to $\delta^{15}N$ fluctuations, and *M. umbilicatum* to be used as an effective "tracer" of N source in the Hawkesbury-Nepean River.

The rate of N turnover in *M. umbilicatum* was measured in order to determine the length of time needed for the algae to equilibrate to their N environment in the field. Under ideal laboratory conditions, N was turned over in 7-8 days, but may be slightly slower under field conditions. For this reason, it was decided to leave the algae in the field for 14 days, to allow them to equilibrate fully with their N environment under variable conditions. All algae were left in the field for this length of time.

Under normal conditions, *M. umbilicatum* expresses a fractionation of approximately –1‰ between N source and the alga. All *M. umbilicatum* $\delta^{15}N$ values measured in the field experiments can therefore be expected to be underestimating the environmental $\delta^{15}N$ by this amount. However, differing environmental conditions, such as light and N concentration between sites can mean that fractionation is enhanced to values of up to –3‰ at some sites (Figures 3.4 and 3.5).

Differences in fractionation due to $NO_3^-$ concentration variability between sites in the Hawkesbury-Nepean River need to be accounted for when interpreting the field data. The mean fractionation by *M. umbilicatum* due to $NO_3^-$ concentrations $\geq 5$ mg.$L^{-1}$ was -2.7 ± 0.2‰ (Figure 3.4). The $\delta^{15}N$ data from sites in the Hawkesbury-Nepean River with a $NO_3^-$ concentration $\geq 5$ mg.$L^{-1}$ were therefore adjusted by this amount.

Light penetration through the water column varies widely between sites in the Hawkesbury-Nepean River due to turbidity differences (NSW EPA, 1994). Increased fractionation due to low light also needs to be taken into account at some sites therefore. The mean fractionation by *M. umbilicatum* due to light one third of available levels was –3.0 ± 0.1‰. (Figure 3.5). The $\delta^{15}N$ data from sites in the Hawkesbury-Nepean River where light intensity at the depth of the algae was substantially reduced by turbidity were therefore adjusted by this amount.

If both high nutrient concentrations and low light levels were present at the same site, their effect was considered to be additive, and the final $\delta^{15}N$ of *M. umbilicatum* algae from these sites was adjusted by the combined fractionation of the two conditions, -5.6%. This simplified adjustment to the possibly complex interactions between light and nutrient concentration is discussed further in Chapter 6.

Temperature does not vary widely between sites in the Hawkesbury-Nepean River and fractionation of N at temperatures between 10 and 25°C was negligible in any case (Figure 3.6). It was therefore determined to be unnecessary to adjust the final $\delta^{15}N$ of *M. umbilicatum* to allow for the influence of temperature.

Salinity at the sites selected in the Hawkesbury-Nepean River ranges from fresh-water to full seawater (NSW EPA, 1994). However, fractionation of N at salinities ranging from 0 to 40 ppt was negligible (Figure 3.7). It was therefore unnecessary to adjust the final $\delta^{15}N$ of *M. umbilicatum* to allow for the influence of salinity.

Adjustments to the final $\delta^{15}N$ of *M. umbilicatum* algae used in the field were carried out where necessary i.e. for data from algae deployed to sites with high nutrient concentrations or low light levels for a significant period of the experiment. These are explained in Section 5.2.2.

# CHAPTER 4: CHARACTERISING THE $\delta^{15}N$ SIGNALS OF THE MAIN SOURCES OF NITROGEN IN THE HAWKESBURY-NEPEAN RIVER.

## 4.1 Introduction

A fundamental process in successfully utilising $\delta^{15}N$ values as a "tracer" in an ecosystem is the characterisation of any possible N sources. This, together with knowledge of any possible fractionations between sources and sink, can make it possible to determine the main sources of N utilised by an organism.

$\delta^{15}N$ values may be widely different between sources or may be very similar. This depends entirely on the number, type and fractionations associated with processes affecting N. As a general rule, the more biological, physical or chemical processes N has undergone since fixation, the greater its $\delta^{15}N$. However, this simplified interpretation is subject to a number of limitations, as discussed in detail in Chapter 2. The $\delta^{15}N$ values for each of the main sources of N in the Hawkesbury-Nepean River were characterised in a series of experiments that are described in this Chapter.

### *4.1.1 Sewage treatment plants*

There are currently 98 licensed Sewage Treatment Plants (STPs) in the Hawkesbury-Nepean catchment. Of these, 43 discharge effluent directly into the river system, while the others irrigate the major part of their effluent to land (NSW EPA, 1993). The major plants in the catchment (see Figure 1.3 for locations) discharge between them approximately 2700 kg of N per day into the river. Much of this N has a distinctively high $\delta^{15}N$ due to the biological and chemical processes that take place in the STPs.

Raw sewage generally has a $\delta^{15}N$ of approximately 6‰ (Spies *et al.*, 1989; Van Dover *et al.*, 1992; Lowe, 1996). This is largely due to the relatively high trophic position of humans (see Section 2.3.1 for a discussion of $\delta^{15}N$ and trophic level). Effluent from STPs can have a $\delta^{15}N$ signal as high as 20, however, due to the processes undergone in the plant (Edwards, 1973; Spies *et al.*, 1989; Heaton, 1996). Wastewater undergoes a large number of processes before being released to the environment, the majority of which discriminate against $^{15}N$, leaving any unreacted substrate in the effluent enriched in the heavier isotope.

Sewage treatment can be divided into three broad stages: primary, secondary and tertiary treatment. Primary treatment generally involves only the removal of particulate matter by simple physical processes such as screening and sedimentation. Secondary treatment removes organic matter by biological processes, and tertiary treatment is aimed at further improving characteristics of the effluent such as N and P loads, clarity and pathogenic microbe/parasite levels (Degremont, 1991b; Barnes, 1995).

Primary treatment has little effect on the amount or form of N present in the effluent, and causes few changes in $\delta^{15}N$ (Degremont, 1991b). Effluent from STPs that treat sewage only to a primary level would therefore be expected to have high N loads and a $\delta^{15}N$ similar to that

of raw sewage (6 - 8‰). Secondary treatment can involve the engineering of microbial populations and their environment to achieve substantial oxidation of $NH_4^+$ to $NO_3^-$, and sometimes the subsequent removal of N by denitrification (Figure 4.1; Degremont, 1991a). These processes can lead to significant reductions in N loads and an accompanying increase in the $\delta^{15}N$ of the effluent to 10 - 20‰. Tertiary treatment may involve further nitrification and denitrification by both biological and chemical processes. Denitrification at this stage is most commonly achieved by increasing pH to >11 by adding lime. This displaces $NH_4^+$ ions and drives off $NH_3$, further enriching the remaining compounds in $^{15}N$ (Degremont, 1991b). This results in an increase in the $\delta^{15}N$ of the treated effluent to levels of up to 30‰ (Edwards, 1973; Spies *et al.*, 1989; Heaton, 1996).

Initially, N is present in sewage primarily as $NH_4^+$ and other organic forms (IWPC, 1975; Degremont, 1991a & b). Secondary treatment converts the bulk of the N to oxidised forms ($NO_3^-$ and $NO_2^-$) and tertiary denitrification converts this to $N_2$ gas, which is lost to the atmosphere. Organic forms of N can be converted to $NH_4^+$ and enter this cycle, or assimilated by bacteria and removed as biomass (Figure 4.2; Degremont, 1991a).

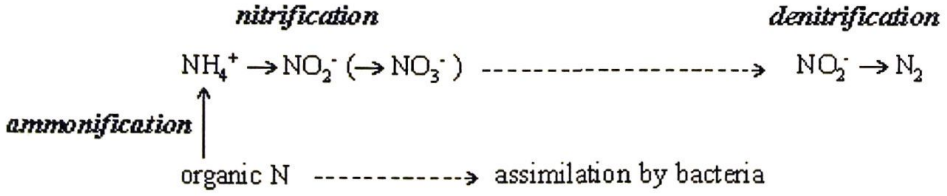



**Figure 4.1**: Transformations of nitrogen in sewage treatment plants.

Nitrification produces $^{15}N$-enriched $NH_4^+$ and $^{15}N$-depleted $NO_3^-$ (Hogberg, 1997). Fractionation for this reaction can be as high as –37‰ (Mariotti *et al.*, 1981). However, in practice, the reaction generally behaves more like a closed system, and this level of fractionation is not expressed (see Section 2.2.3 for discussion). Denitrification can produce fractionation of up to -31‰, as the lighter isotope is preferentially driven off as gas (Blackmer and Bremner, 1971; Mariotti *et al.*, 1981; Smith and Duff, 1988). The more N is removed from the effluent as $N_2$ gas, the higher the $\delta^{15}N$ of the remaining N compounds becomes. It is this process which produces the very high $\delta^{15}N$ signal of sewage effluent.

All of the major STPs discharging into the Hawkesbury-Nepean River treat effluent to tertiary level. A number of them also denitrify the effluent before release. The exact manner of treatment differs between plants, but the main methods utilised are activated sludge processes, lagoons and trickling filters (Table 4.1).

Trickling filters are surfaces designed to allow a biological film to develop. Wastewater is then slowly passed over these films, and $NH_4^+$ is oxidised to $NO_3^-$ / $NO_2^-$ (Degremont, 1991a). Lagooning allows these processes to take place in an open pond system, which is often aerated. In some systems, the remaining sludge is periodically decanted (Sewards and Williams, 1995). Activated sludge processes bring the sewage into contact with a bacterial floc in the presence of oxygen. Water is then separated from the floc (clarification; IWPC, 1975; Degremont, 1991a). Continuous flow activated sludge processes include an additional step, where $NO_3^-$-containing liquid is circulated to anaerobic areas where it contacts the incoming sewage. This reduces the $NO_3^-$ to $N_2$ gas and simultaneously oxidises some of the incoming wastewater (Raper, 1995).

**Table 4.1**: Types of waste treatment processing at the major sewage treatment plants discharging into the Hawkesbury-Nepean River.

| STP | Discharge point | Process type | Level of effluent treatment |
|---|---|---|---|
| ***Mt Riverview*** | Lapstone Creek | Trickling filter | Tertiary (no nutrient removal) |
| ***Penrith*** | Boundary Creek | Biological nutrient removal | Tertiary (N and P removal) |
| ***Quakers Hill*** | Eastern Creek | Intermittently decanted aerated lagoon | Tertiary (N and P removal) |
| ***Riverstone*** | Eastern Creek | Continuous flow activated sludge | Tertiary (P removal only) |
| ***St. Marys*** | South Creek | Continuous flow activated sludge | Tertiary (P removal only) |
| ***Castle Hill*** | Cattai Creek | Continuous flow activated sludge | Tertiary (P removal only) |
| ***Round Corner*** | Cattai Creek | Package activated sludge | Tertiary (no nutrient removal) |

All these treatment processes increase the $\delta^{15}N$ of effluent markedly from the raw sewage entering the plant. STPs with an additional N removal step would be expected to have effluent with particularly high $\delta^{15}N$. The $\delta^{15}N$ of the effluent of the main STPs discharging into the Hawkesbury-Nepean River was characterised, and the results are outlined in Section 4.3.1.

### *4.1.2 Agricultural run-off*

Agriculture and urban development is extensive in the Hawkesbury-Nepean catchment, and is quite intensive in some areas (see Section 1.3.1). Diffuse source pollution is associated with agricultural and urban activities when contaminated surface run-off waters discharge into watercourses. The most significant sources of this type of pollution in the Hawkesbury-Nepean catchment are believed to be agricultural run-off from activities such as pasture, market gardening, pig and poultry farming, dairying and turf farms (NSW EPA, 1993). It is beyond the scope of this thesis to identify individual diffuse sources of pollution in the catchment. Instead, an effort has been made to identify the main diffuse sources, and characterise the combined $\delta^{15}N$ of these sources to the river.

Market gardens are concentrated in the South Creek, Woolondilly, Cattai and Richmond/Windsor areas. Turf farming is carried out predominantly on the floodplain of the middle Hawkesbury River and on the floodplain of the Nepean River between Camden and Penrith (see Figures 1.2 and 1.3; NSW EPA, 1993). Piggeries are concentrated in the Woolondily and Baulkham Hills areas. Poultry raising is concentrated in the Gosford, Penrith and Baulkham Hills areas. Dairying is also a widespread activity in the catchment, with most dairies found in the Camden region (see Figures 1.2 and 1.3; NSW EPA, 1993).

Intensive agricultural activities, such as piggeries, market gardens and turf farms appear to have the greatest potential to pollute the Hawkesbury-Nepean River (NSW EPA, 1993). Run-off arising from market gardens, crops and turf farms consists mainly of nutrients, agricultural chemicals and sediment. In contrast, run-off from intensive animal activities such as feedlots, piggeries and chicken farms will consist of primarily of animal wastes.

Intensive horticultural activities will lead to run-off with a nitrogen content primarily composed of artificial fertilisers. Commercial fertilisers usually contain nitrogen in the form of ammonium or potassium nitrate, and have a $\delta^{15}N$ between –3 and +3‰ (Section 2.2; Heaton, 1986; Hopkins *et al.*, 1998). Once in the soil however, these N compounds may be transformed by a number of processes, altering their $\delta^{15}N$ markedly. Microbial populations will uptake and assimilate $NH_4^+$ and $NO_3^-$. Nitrifying bacteria may oxidise $NH_4^+$ to $NO_3^-$, a process which can result in fractionations of up to +9‰ (Handley and Raven, 1992; Hopkins *et al.*, 1998). Some $N_2O$ gas may also be produced by these bacteria under certain conditions (Hopkins *et al.*, 1998). Denitrification (the production of $N_2$ gas from $NO_3^-$ or $NO_2^-$) in soils is carried out by a wide range of facultative anaerobes and fungi. This process may result in discrimination of up to +33‰ (Handley and Raven, 1992).

As well as fertiliser-derived N, agricultural soils may contain N sourced from atmospheric deposition and mineralisation, the release of N compounds from organic remains or wastes (Hopkins *et al.*, 1998). These will all have characteristic $\delta^{15}N$ values. However, the bulk of N in soils used for intensive agriculture is usually derived from inorganic fertiliser.

Gaseous N loss from soils is a significant process, and may lead to marked changes in the $\delta^{15}N$ of the remaining N compounds. Up to 86% of applied fertiliser N may be lost to the atmosphere as $NH_3$, $N_2O$ or $N_2$ gas (Freney and Black, 1987). The amount of N lost to the atmosphere is heavily dependent on moisture, with the majority of $N_2O$ lost from the soil within a few hours of wetting (Jorgensen *et al.*, 1998). $NH_4^+$ volatilisation may also enrich the top layers of soil in $^{15}N$, as the lighter isotope is preferentially lost (Handley and Scrimgeour, 1997). This is a pH-dependent reaction, with more $NH_4^+$ lost from high pH soils (Freney and Black, 1987; Handley and Scrimgeour, 1997).

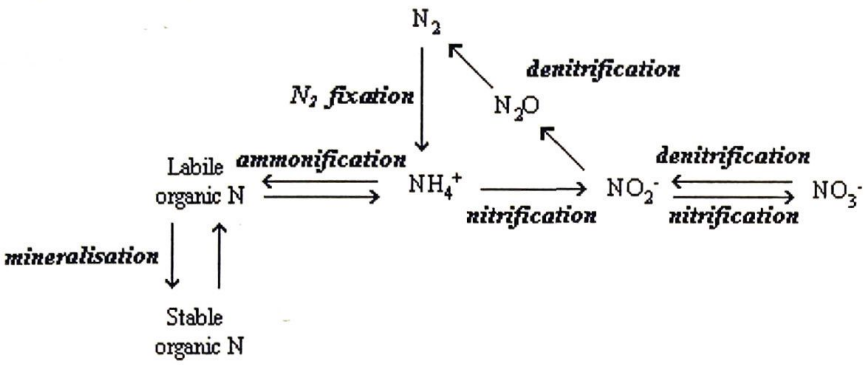



**Figure 4.2**: Microbial transformations of nitrogen in soil (adapted from Hopkins *et al.*, 1998).

All of these processes contribute to the final $\delta^{15}N$ of run-off from cultivated fields. Nitrate in run-off from these types of agricultural soils has a $\delta^{15}N$ usually in the range of –3.5 to +8‰ (Bremner and Tabatai, 1973; Herbel and Spaulding, 1993; Komor and Anderson, 1993). Nitrate and $NH_4^+$ in run-off of this type have been found to have a combined $\delta^{15}N$ of +1 to +6‰ (Kreitler, 1979; Hopkins *et al.*, 1998).

Run-off from intensive animal farming will consist largely of animal wastes. Run-off may be high in various N compounds, particularly $NH_4^+$, and will have a relatively high $\delta^{15}N$. Animal waste will have a $\delta^{15}N$ of +4 to +13‰, depending on the foodstuffs provided (see Section 2.3.1). Waste from intensive animal production is often held in ponds or irrigated onto agricultural land. Denitrification in ponds may further increase the $\delta^{15}N$ of animal waste, while irrigated wastes are subject to the same soil processes as other N sources. Run-off derived primarily from animal waste has been found to have a $\delta^{15}N$ ranging from +8 to +22‰ in previous studies (Kreitler, 1973; Herbel and Spaulding, 1993; Komor and Anderson, 1993; Hopkins *et al.*, 1998).

It was not attempted in this study to distinguish between run-off derived from intensive horticultural and animal production. Instead, a characterisation of the overall $\delta^{15}N$ of run-off from all types of land, including urban and forested areas was attempted. The $\delta^{15}N$ of run-off from representative areas into the Hawkesbury-Nepean River was characterised, and the results are outlined in Section 4.3.2.

### *4.1.3 A further factor: Sediment*

Sedimentary processes play a very important part in the budgets for carbon, phosphorus and nitrogen in aquatic ecosystems. Although they are not in themselves a significant source of N, sediments store, recycle and remove large amounts of N in the Hawkesbury-Nepean River (NSW EPA, 1994). Most of the processes taking place in the sediment are mediated by specialist bacteria, and hence are likely to involve a level of fractionation (see Section 2.2.2). Nitrogen released from the sediments will therefore reflect both the $\delta^{15}N$ of its source and any discriminatory processes undergone in the sediment.

Nitrogen enters the sediment as particulate organic N from decaying organisms and other organic sources (Sigleo and Macko, 1985; Webster, 1998). Particulate organic N is hydrolysed to dissolved organic N. This is then ammonified to $NH_4^+$. $NH_4^+$ is nitrified to $NO_3^-$, then denitrified to $N_2$ by bacteria (Figure 4.3; Plenet *et al.*, 1995; Webster, 1998).

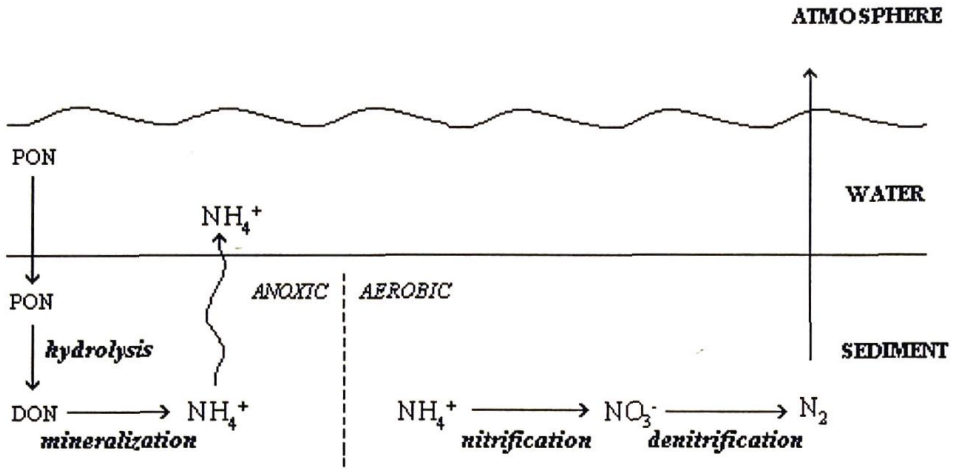



**Figure 4.3**: Transformations of nitrogen in aquatic sediments (Note: the aerobic-anaerobic interface has been drawn as a vertical rather than horizontal interface for clarity of presentation of the processes.)

Ammonium released from the sediments would be expected to be significantly enriched in $^{15}N$ (Cifuentes *et al.*, 1988). Both nitrification and denitrification by bacteria discriminate against the heavier isotope, leaving any non-reacted N compounds enriched in the heavier isotope (Mariotti *et al.*, 1981). Sedimentary $\delta^{15}N$ has been reported to range between +5 and +13 ‰ in other studies (Pennock, 1987; Cifuentes *et al.*, 1988; Mulholland and Olsen, 1991). This value reflects the $\delta^{15}N$ of original source N and the additional enrichment taking place in the sediment.

Removal of N from the aquatic ecosystem by denitrification is an extremely important process, with up to 90% of the N entering the sediment released to the atmosphere as $N_2$ gas in some systems (Berelson *et al.*, 1996), although this process has not been quantified for the Hawkesbury-Nepean. The rate of denitrification is dependent on the concentration of $NO_3^-$ in

the water column and the availability of oxygen (Heggie *et al.*, 1998; Skyring, 1998; Webster, 1998). If oxidation of $NH_4^+$ is prevented by a lack of oxygen in the sediment (a consequence of eutrophication), then nitrification and denitrification cannot occur (Berelson *et al.*, 1996; Heggie *et al.*, 1998). Under anoxic conditions, the water column will then become a sink for the N effluxed from the sediments as $NH_4^+$. Denitrification efficiency has also been shown to be a function of the amount of sedimentary organic carbon mineralised (Heggie *et al.*, 1998). Increased C loads to the sediment reduce its denitrification capacity. Eutrophication of the water column commonly leads to algal blooms (see Section 1.1), which add large amounts of C to the sediment when they decompose. Together with the reduced $O_2$ also associated with eutrophication, this can result in sediments releasing large amount of N to the water column.

Under the right conditions, the sediment can be a significant source of N, releasing stored N compounds to the water column rather than reducing N loads via denitrification. It is beyond the scope of this thesis to investigate the cycling of N in the sediments in detail, as the main aim of this project is to determine only the original major sources of N contributing to eutrophication. Although N released from the sediments has originated elsewhere, the sediment is its immediate source. It was therefore decided to characterise the $\delta^{15}N$ of N arising from the sediment under anoxic conditions. These results are outlined in Section 4.3.3.

### *4.1.4 Aims: Isotope signatures of major sources of nitrogen*

The aim of this project was to determine the isotope signatures of the main sources of N contributing to the eutrophication of the Hawkesbury-Nepean River. Possible major sources of N in the catchment include agricultural run-off, effluent from sewage treatment plants and N recycled from the sediments (NSW EPA, 1993; NSW EPA 1994; AWWA, 1998). Characteristic $\delta^{15}N$ signals for each of these were established, and are presented in this Chapter. These values were then used to determine the relative contributions of each of the major sources of N to the Hawkesbury-Nepean River in Chapter 5.

## 4.2 Materials and Methods

### *4.2.1 General experimental conditions*

*Microdictyon umbilicatum* algae were cultured as usual for these experiments (Section 3.2.1). However, the salinity of the growth medium was adjusted to approximately match that of the intended field site of each group of algae (see Section 3.7.1 for details on salinity adjustment methods). Half the *M. umbilicatum* algae were cultured in high $\delta^{15}N$ nutrient, and half in low $\delta^{15}N$ nutrient to provide a control for the turnover of N during the experiments (Section 3.2.1). Algaewere deployed in the river in custom-made apparatuses designed to allow them contact with the water column but prevent them from being washed away (Figure 4.4).

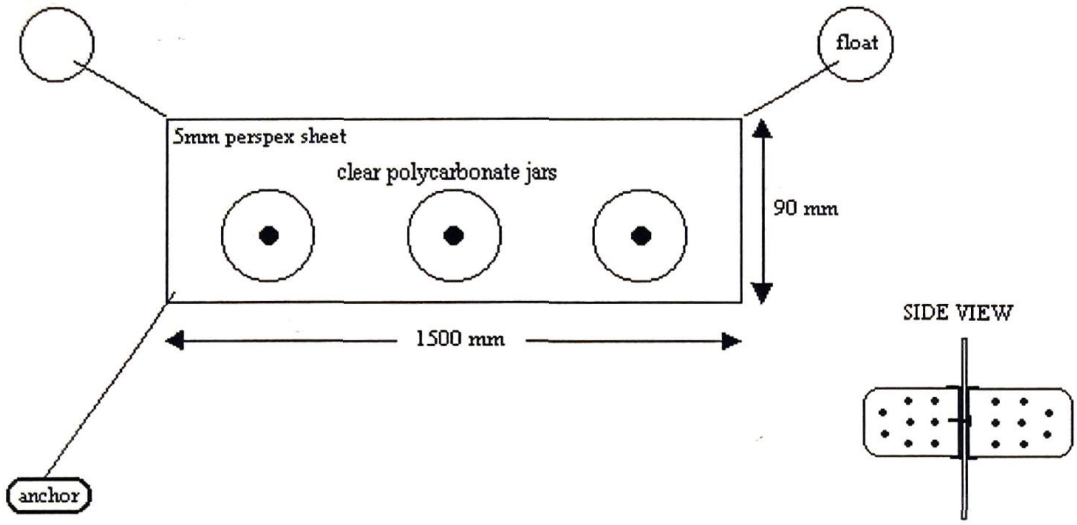



**Figure 4.4**: Apparatus used to deploy *Microdictyon umbilicatum* algae in the Hawkesbury-Nepean River.

Each apparatus holds 6 algae, each contained within a separate compartment. Compartments were clear polycarbonate jars arranged on either side of a clear Perspex sheet. This allowed maximum light penetration to the algae. Floats were used and adjusted to maintain the jars at a depth of approximately 15 cm. Water flow-through was facilitated by a series of holes made in each jar with a soldering iron. Holes were approximately 3 mm in diameter, large enough to allow an unrestricted flow of water, yet small enough to ensure the algae remained in the apparatus.

In the field, algae were transferred from 500 mL polyurethane jars containing their growth medium directly to the apparatuses. Three algae from high $\delta^{15}N$ growth media and 3 from low $\delta^{15}N$ growth media were randomly allocated to each apparatus. Algae were left in the field for 14 days before collection.

After collection, algae were immediately removed from the apparatus and rinsed in clean water from the laboratory (with the same approximate salinity as the field site). Algae were stored in individual 500 mL polyurethane jars containing clean water for transport back to the laboratory. Alga biomass was recorded as wet weight at the beginning and end of the two-week period, and replicates were discarded if their biomass had not increased by at least 50%. This was to ensure that algae had equilibrated to their new environment. Generally, it was found that algae that had not increased in biomass had been affected by problems with the experimental apparatus (for example, water flow into the chamber had been interrupted by a blockage, or the chamber had emerged from the water). In the laboratory, algae were rinsed again in distilled water and any epiphytes or unhealthy sections of the alga were removed. Samples were then prepared for analysis of nitrogen isotope composition as usual (Section 3.2.2).

A total of 12 replicate algae were deployed at each site at each time, 6 per apparatus. Apparatuses were < 50m apart. Results from both apparatuses were pooled for the graphs presented here. Algae cultured in both high ($\delta^{15}N$ =10‰; Section 3.2.1) and low ($\delta^{15}N$ =5; Section 3.2.1) $\delta^{15}N$ nutrients were used to ensure N turnover (Sections 3.2.1 and 4.2). High and low $\delta^{15}N$ algae were allocated randomly, 3 of each per apparatus (STP and agricultural run-off) or aquarium (sediment).

### *4.2.2 Sewage treatment plants*

In order to define a characteristic $\delta^{15}N$ for sewage effluent discharged into the Hawkesbury-Nepean River, algae were deployed in the river at sites near STP outfalls, where the bulk of N is sewage-derived (Figure 4.5). Some sites were further from outfalls than others for logistical and access reasons, but efforts were made to determine that the bulk of N at these sites would reasonably be expected to be sewage derived and not from other sources.

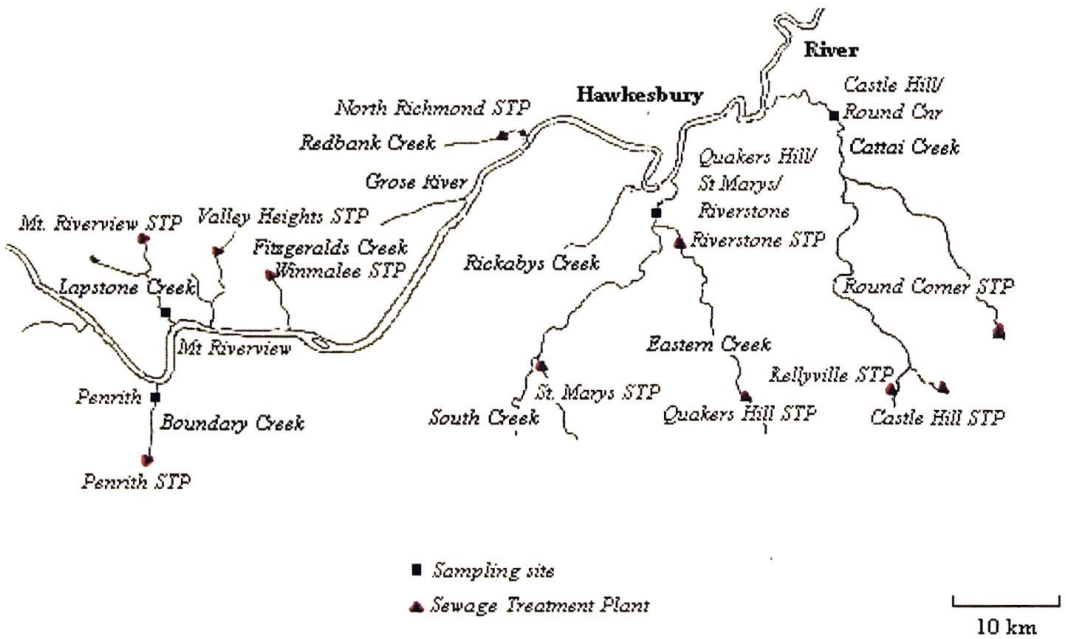



**Figure 4.5**: Sampling sites for the collection of representative $\delta^{15}N$ data on sewage treatment plant discharge.

The effluent from some STPs was characterised as one measurement (i.e. Quakers Hill/St Marys/Riverstone and Castle Hill/Round Corner; Figure 4.5) as the effluent from these STPs enters the main river together. The aim of this part of the experiment was to characterise these three STPs. Although they may well have been some processes (for example, cycling through sediments, denitrification) occurring in the creek and affecting $\delta^{15}N$ before the sample site, these were not deemed to be important enough to affect the overall aim of this experiment, which was to establish a "characteristic" $\delta^{15}N$ for STP effluent. Replicate STP effluent characterisation experiments were carried out in September 1997, July 1998 and March 1999.

### *4.2.3 Agricultural run-off.*

In order to define a characteristic $\delta^{15}N$ for agricultural run-off entering the Hawkesbury-Nepean River, algae were deployed in the river at sites where the bulk of N is derived from run-off and there is minimal input from STPs (Figure 4.6).

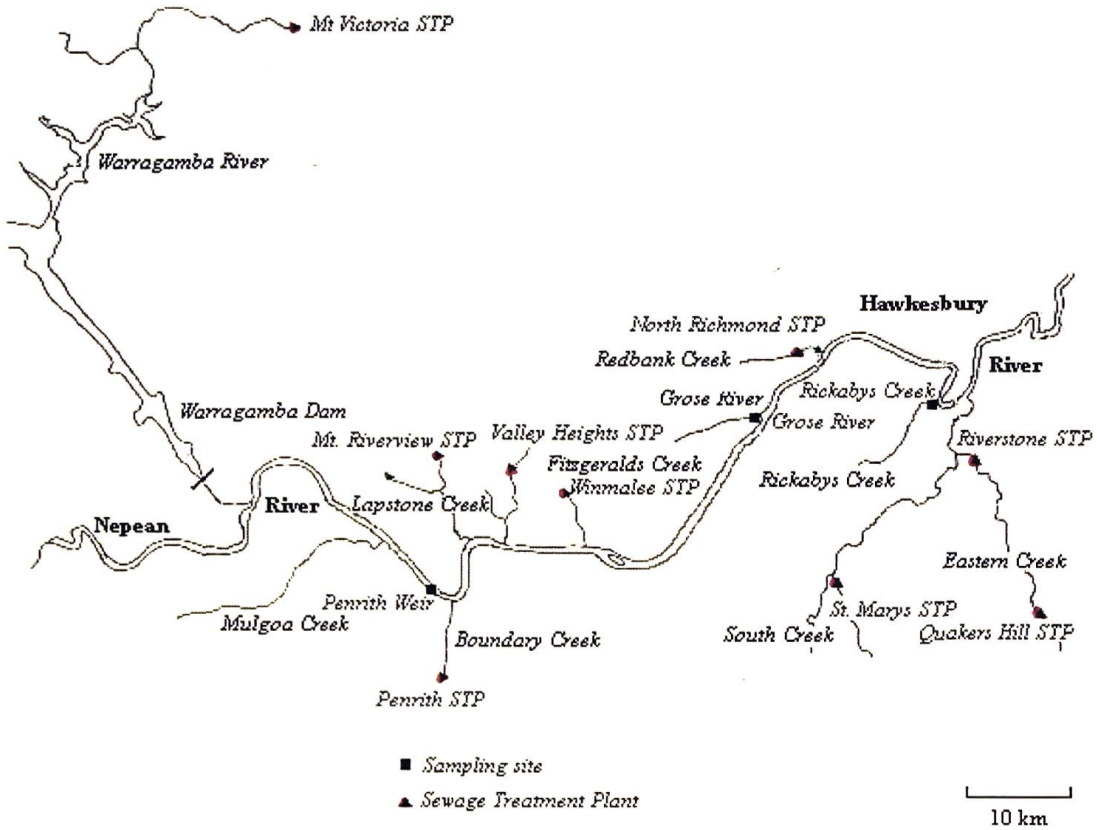



**Figure 4.6**: Sampling sites for the collection of representative $\delta^{15}N$ data on agricultural run-off.

Agricultural run-off characterisation experiments were carried out in April 1997, July 1998 and February 1999. Results from these experiments are presented in Section 4.3.2.

### *4.2.4 Sediment*

In order to define a characteristic $\delta^{15}N$ of sediment in the Hawkesbury-Nepean River, algae were grown in aquariums containing pore water from sediment sampled from sites along the length of the river (Figure 4.7). Sites were allocated in order to be representative of the different $\delta^{15}N$ conditions present along the length of the river.

In order to obtain N compounds from the sediment, cores were taken from the sediment at each of the sites selected (Figure 4.7). Cores were taken to a depth of approximately 40cm with a length of PVC pipe (diameter 21cm). Five cores were taken within a 50m radius at each site. Sediments were immediately transferred from the coring device to 1L polyurethane jars and transported back to the laboratory. Once in the laboratory, sediments were left in the sealed polyurethane jars for 1 week to allow the samples to become anoxic. This mimicked conditions in the field where N compounds would be expected to efflux from the sediment into the water column (see Section 4.1.3). Pore water was then removed from the samples by centrifugation, and the solid portion discarded.

All 5 pore water samples from each site were pooled. The pore water was added to filtered seawater and this was used as the nutrient media for *M. umbilicatum* kept under otherwise normal conditions for two weeks (Section 3.2.1). Pore water samples were kept in sealed 500mL polyurethane jars at -20°C until required. Pore water nutrient media was renewed using this stored portion every two days for the duration of the experiment.

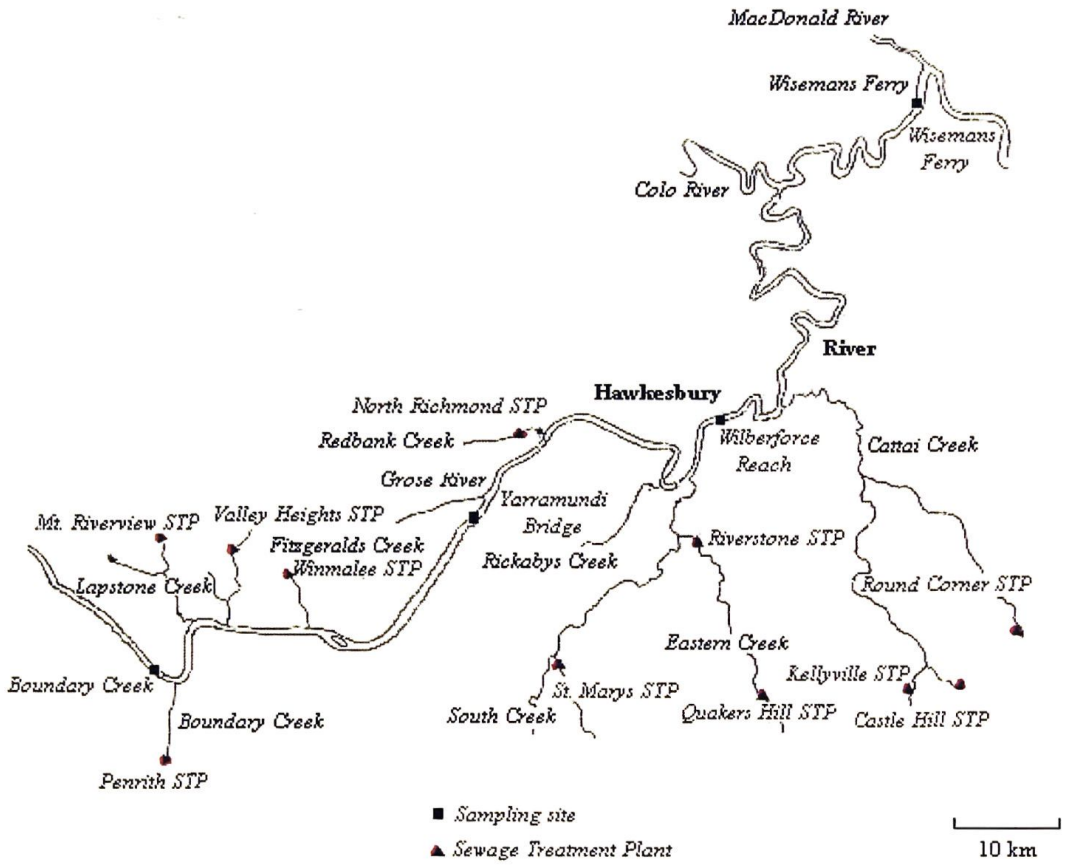



**Figure 4.7**: Sampling sites for the collection of representative $\delta^{15}N$ data on sediment.

Replicate sediment characterisation experiments were carried out in September 1997, July 1998 and March 1999. Results from these experiments are presented in Section 4.3.3.

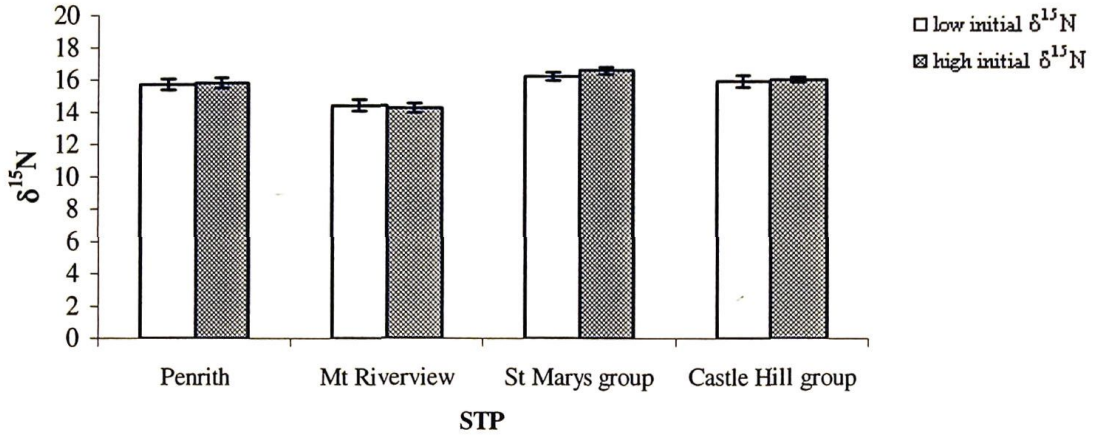
## 4.3 Results

### *4.3.1 Sewage treatment plants*

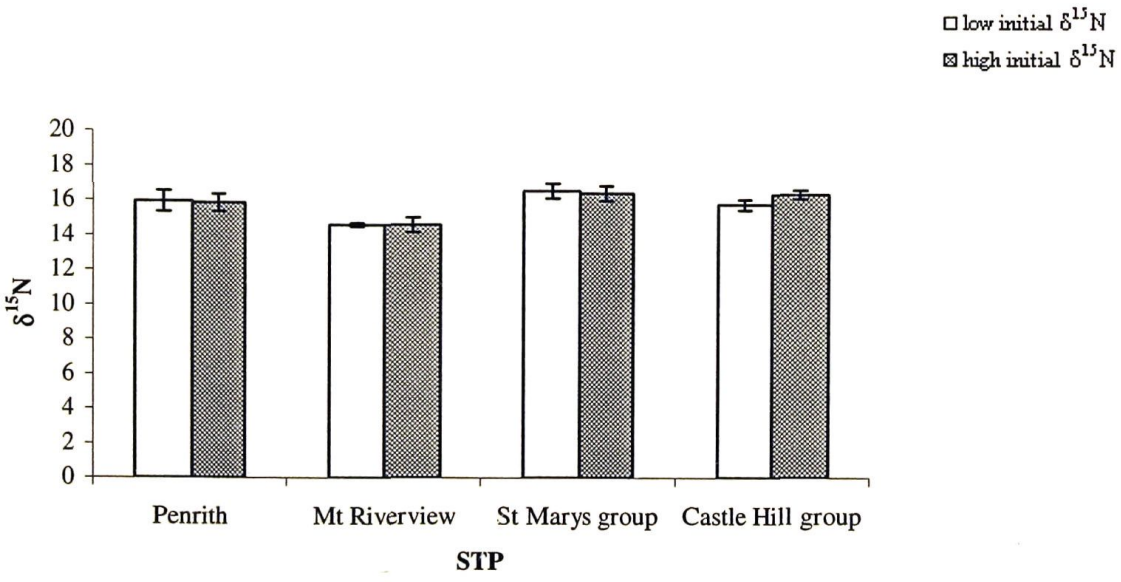
The mean $\delta^{15}N$ of effluent of *M umbilcatum* algae grown at river sites dominated by sewage effluent, was 15.6 ± 1.6‰ (Figure 4.8). Although there were differences in mean effluent $\delta^{15}N$ between STPs, there was no clear pattern to these, apart from consistently lower values at the Mt. Riverview plant, and all values fell within the range of 14.0 to 17.1‰. Values were also consistent between the September 1997, July 1998 and March 1999 experiments (Figure 4.8).

No allowance was made for the effect of fractionation by *M. umbilicatum*, or variation in discrimination caused by changes in environmental conditions (see Chapter 3) in the results presented below. The possible effect of these factors is discussed in Section 4.4.1. Results are presented here as mean $\delta^{15}N$ of *M. umbilicatum* algae deployed in the river. An internal control for N turnover was provided by the "high" and "low" initial $\delta^{15}N$algae, and these results are presented for each experiment as parallel results. N turnover was completed within the two-week experimental period, as there is no significant difference between initial "high" and "low" treatments. Variance between replicates is also very low, even when data from both apparatuses were pooled (Figure 4.8).

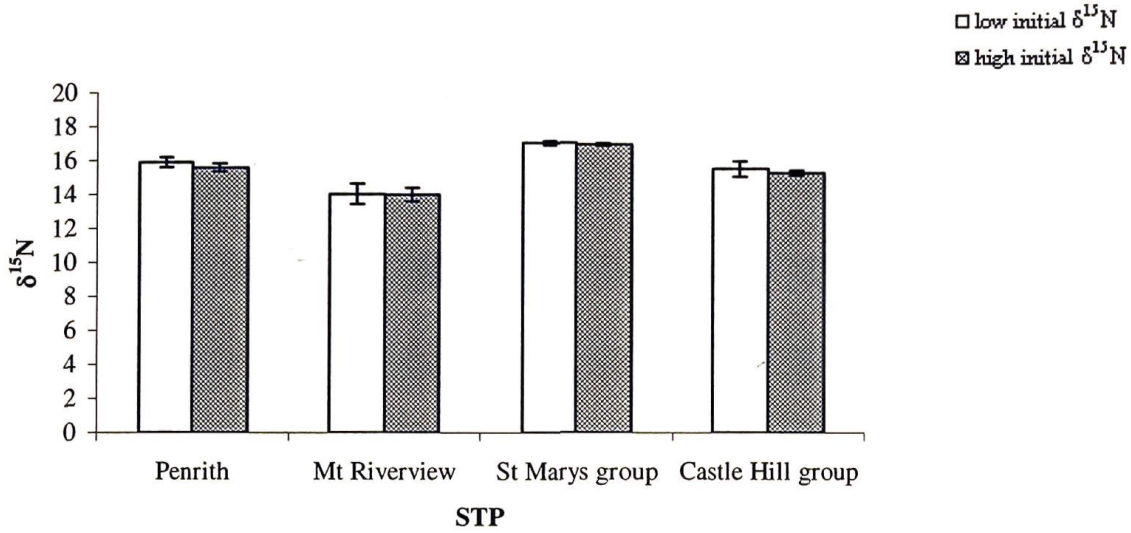
**A**: September 1997.




**B**: July 1998.



C: March 1999.



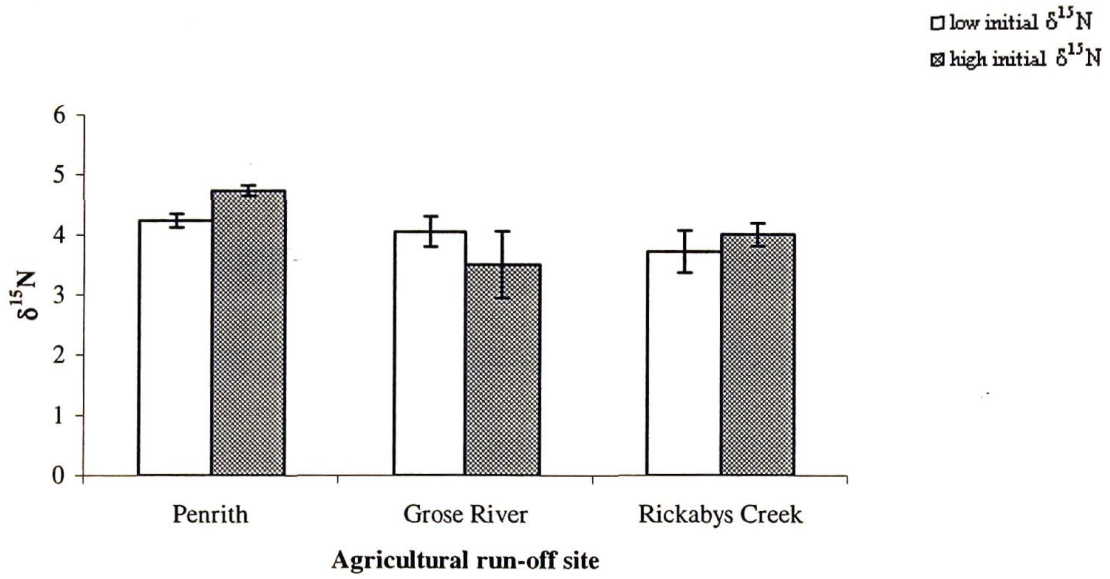

**Figures 4.8**: Mean $\delta^{15}N$ measured by *Microdictyon umbilicatum* algae grown in STP effluent (values are mean $\delta^{15}N \pm$ SD (‰) from the 12 replicate algae at each location).

### *4.3.2 Agricultural run-off*

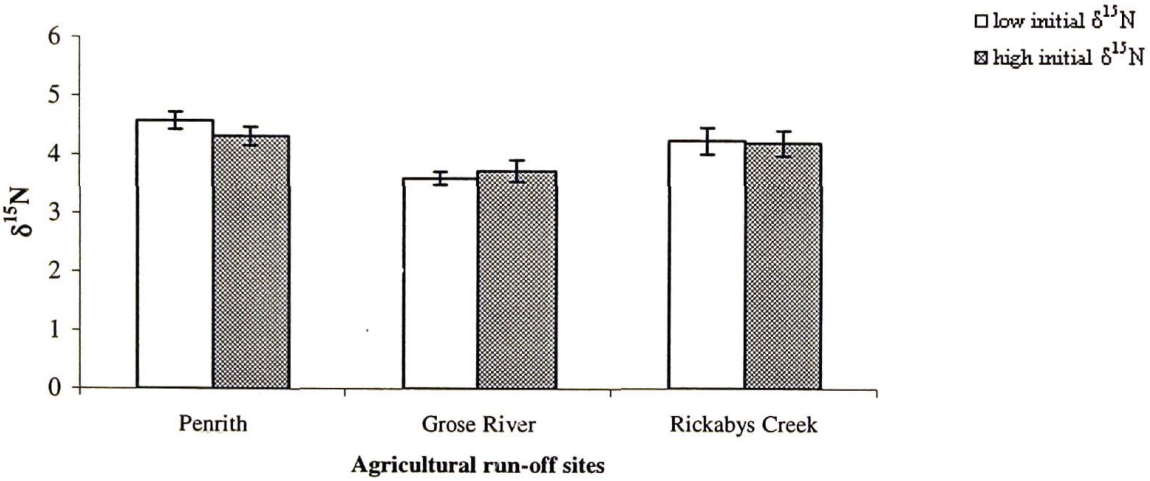
The mean $\delta^{15}N$ *M. umbilicatum* algae grown at sites in the river dominated by agricultural run-off, was 4.1 ± 1.1‰ (Figure 4.9). Although there were differences in mean $\delta^{15}N$ between sites, there was no clear pattern to these, and all values fell within the range of 3.4 to 5.3‰. Values are consistent between the April 1997, July 1998 and February 1999 experiments (Figure 4.9).

No allowance has been made for the effect of fractionation by *M. umbilicatum*, or variation in discrimination caused by changes in environmental conditions (see Chapter 3) in the results presented below. The possible effect of these factors is discussed in Section 4.4.2. Results are presented here as mean $\delta^{15}N$ of *M. umbilicatum* algae deployed in the river. An internal control for N turnover was provided by the "high" and "low" initial $\delta^{15}N$ algae. N turnover was completed within the two-week experimental period, as there is no significant difference between initial "high" and "low" treatments. Variance between replicates was also very low, even when the results from both apparatuses were pooled (Figure 4.9).

**A**: April 1998.




**B**: July1998.



C: February 1999.

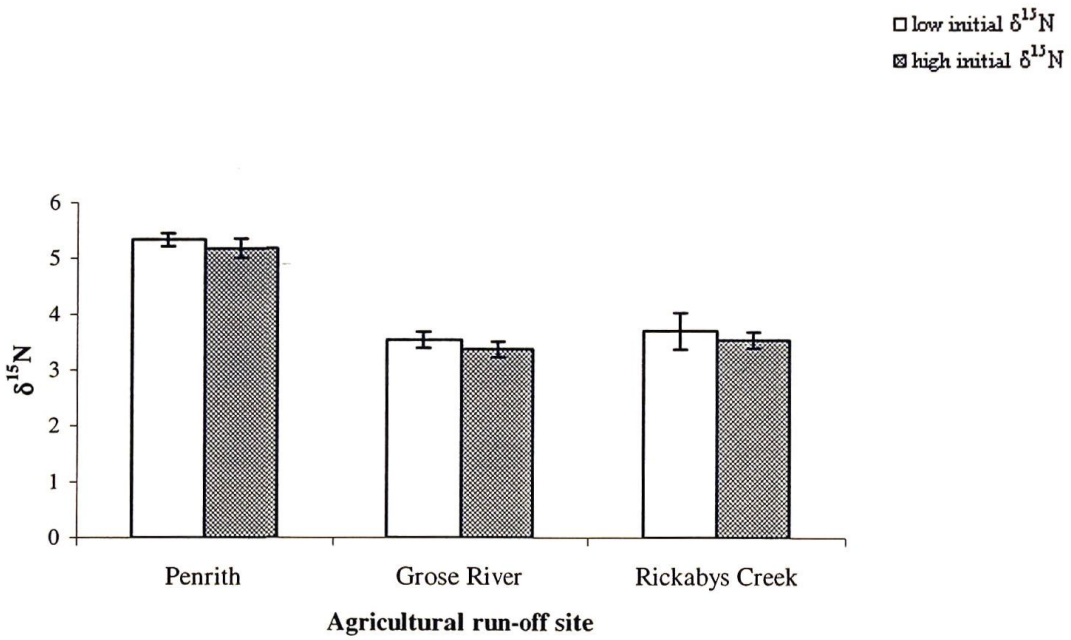


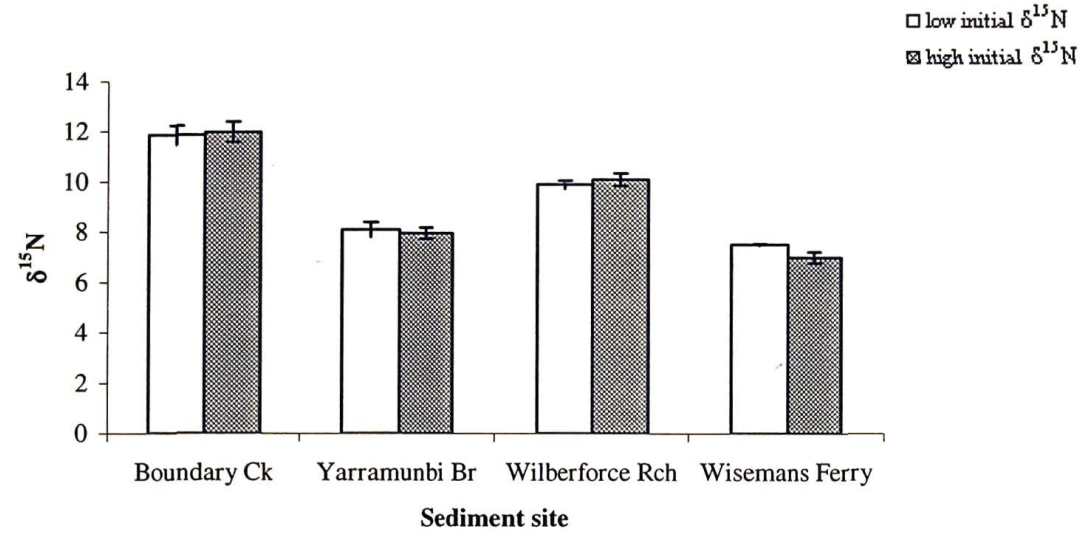

**Figure 4.9**: Mean $\delta^{15}N$ measured by *Microdictyon umbilicatum* algae grown in agricultural run-off (values are mean $\delta^{15}N \pm$ SD (‰) for the 12 replicate algae at each location).

### *4.3.3 Sediment*

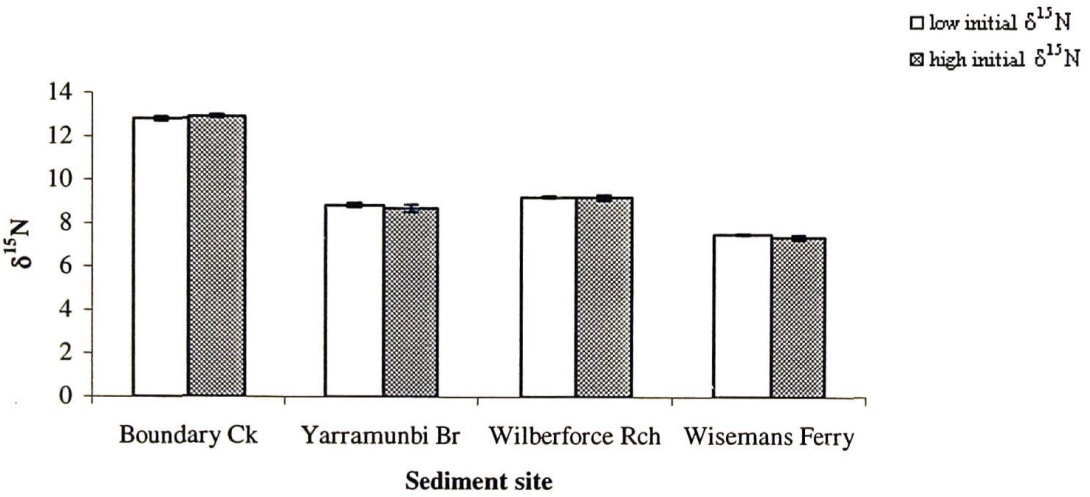
The mean $\delta^{15}N$ of *M. umbilicatum* grown in sediment pore water, was 9.3 ± 3.5‰ (Figure 4.10). There were differences in the mean $\delta^{15}N$ between sites but without any clear pattern or trends. Variance was larger in this experiment than for the other characterisation experiments, but all values fell within the range of 6.9 to 12.9‰. There were no clear differences between values from the September 1997, July 1998 and March 1999 experiments (Figures 4.7a, b and c respectively).

No allowance was made for the effect of fractionation by *M. umbilicatum* in the results presented below. Light levels were kept high enough during the experiments to exclude fractionation (see Section 3.4.3), however, nutrient concentrations could not be controlled. The possible effect of nutrient concentration is discussed in Section 4.4.3. Results are presented here as mean $\delta^{15}N$ of *M. umbilicatum* algae grown in the pooled pore water from sediment collected at each site. An internal control for N turnover was provided by the "high" and "low" initial $\delta^{15}N$ algae. N turnover was completed within the two-week experimental period, as there was no significant difference between initial "high" and "low" treatments. Variance between replicates is also very low (Figure 4.10).

**A**: September 1997.




**B**: July 1998.



C: March 1999.

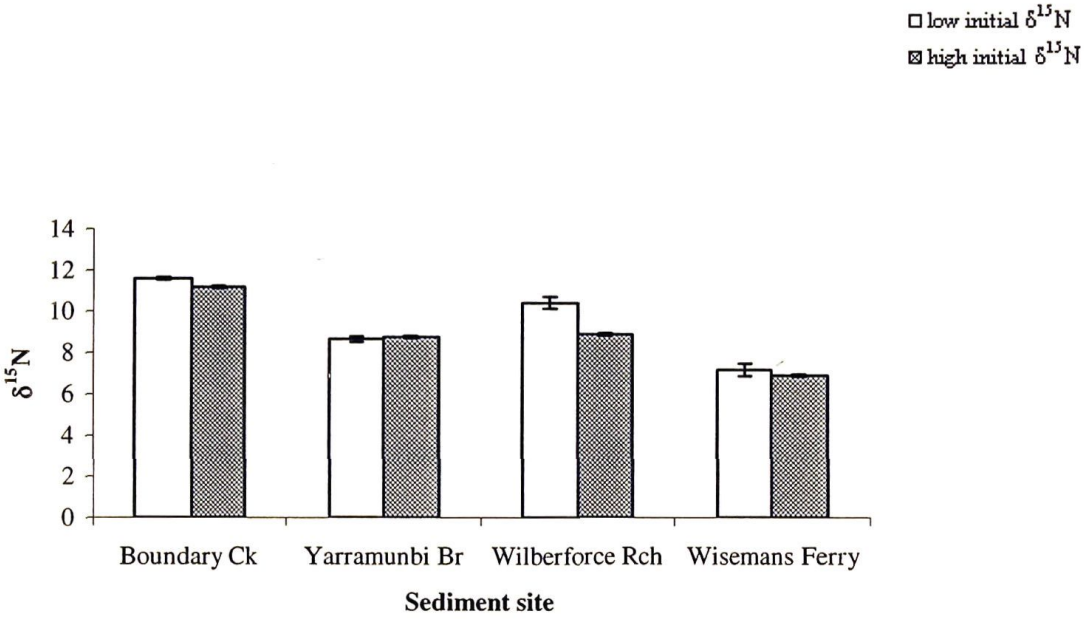



**Figure 4.10**: Mean $\delta^{15}N$ measured by *Microdictyon umbilicatum* algae grown in sediment pore water (values are mean $\delta^{15}N \pm$ SD (‰) from the 6 replicate algae at each location).

## 4.4 Discussion

### *4.4.1 Sewage treatment plants*

Sewage Treatment Plants discharging effluent into the Hawkesbury-Nepean River have a distinctly high $\delta^{15}N$ of consistently greater than 14.0‰. This high $\delta^{15}N$ is due to the processing of N wastes within the STPs, which will enrich the N compounds remaining in the effluent in $^{15}N$. In particular, dentrification, as carried out by the Penrith and Quakers Hill plants, results in very high $\delta^{15}N$ (Mariotti *et al.*, 1981).

There is some difference between STPs, with effluent from Mt Riverview consistently having a lower mean $\delta^{15}N$ than that from the other plants (Figure 4.8). This may be due to slight differences in the treatment of wastewater at each STP. All of the STPs characterised treat effluent to a tertiary level however (Table 4.1), and would be expected to have approximately similar $\delta^{15}N$ values, so it is unknown as to why this trend has occurred. Variation (measured by standard deviation) over time was greater than variation between STPs, indicating that differences in treatment processes between plants were less important to the average $\delta^{15}N$ value than temporal variability. .

Corrections were not made for the influence of fractionation by *M. umbilicatum* or the effect of environmental factors on the level of discrimination in the results presented in Section 4.3.3. ). Nitrate concentrations at all of the sites sampled for characteristic STP effluent $\delta^{15}N$ were well below the level found to cause discrimination (5mg.$L^{-1}$, Section 3.5) at all times. Turbidity at the sites chosen was low, and was not judged to be likely to reduce light levels enough to induce discrimination by *M. umbilicatum.* Discrimination by *M. umbilcatum* would therefore be limited at all sites to the normal level for this alga of –1‰ from source $\delta^{15}N$.

The aim of this experiment was not to distinguish between different STPs, but to identify a mean "characteristic STP $\delta^{15}N$" for use in identifying the main sources of N contributing to the eutrophication of the Hawkesbury-Nepean River. Although $\delta^{15}N$ values differed between STPs (Figure 4.8), a mean "characteristic STP $\delta^{15}N$" could be determined. Over the three times and four STP groups sampled, the mean $\delta^{15}N$ of STP effluent was found to be in the range of 14.0 to 17.0‰. The mean value of 15.6‰ was used as a "characteristic STP $\delta^{15}N$" when interpreting the field results in Chapter 5.

### *4.4.2 Agricultural run-off*

Run-off from agricultural land into the Hawkesbury-Nepean River has a relatively low $\delta^{15}N$, consistently < 5.2‰. This $\delta^{15}N$ is a product of a combination of animal wastes and inorganic fertiliser from the intensive horticulture and animal production in areas in the catchment, and any subsequent fractionations undergone by these compounds before entering the river.

There appears to be some difference between sites, with run-off measured at Penrith being generally higher than run-off measured at Grose River and Rickabys Creek (Figure 4.9). However, there is no statistical difference between the sites. Any differences may be due to some differences in the type of activity, and hence composition of the run-off between these sites. Penrith has a concentration of piggeries, which may contribute a greater load of animal wastes to run-off into the river than at the other two sites. It would be expected that run-off with a higher proportion of animal wastes would have a higher $\delta^{15}N$ than run-off containing primarily N derived from inorganic fertilisers (Komor and Anderson, 1993; Hopkins *et al.*, 1998). However, this difference between sites was within the limits of variance seen within each site, and was therefore of little significance. Variance over time was also greater than

variance between sites, indicating that differences in the composition of run-off between sites were not particularly important.

Corrections were not made for the influence of fractionation by *M. umbilicatum* or the effect of environmental factors on the level of discrimination in the results presented in Section 4.4.3. Nitrate concentrations at all of the sites sampled for run-off $\delta^{15}N$ were well below the level found to increase discrinination (Section 3.5) at all times. Turbidity at the sites chosen was low, and was not judged to be likely to reduce light levels enough to induce discrimination. Discrimination by *M. umbilicatum* would therefore be limited to the usual –1‰ from source $\delta^{15}N$.

The aim of this experiment was not to distinguish between agricultural run-off from different sites, but to identify a mean "characteristic agricultural run-off $\delta^{15}N$" for use in identifying the main sources of N contributing to the eutrophication of the Hawkesbury-Nepean River. Although $\delta^{15}N$ values differed between sites (Figure 4.9), a mean "characteristic agricultural $\delta^{15}N$" value could be determined. Over the three times and sites sampled, the mean $\delta^{15}N$ of agricultural run-off was found to be in the range of 3.4 to 5.3‰. The mean value of 4.1‰ was used as a "characteristic agricultural run-off $\delta^{15}N$" when interpreting the field results in Chapter 5.

### *4.4.3 Sediment*

Nitrogen recycled from sediments in the Hawkesbury-Nepean River had a $\delta^{15}N$ in the range of 6.9 to 12.9‰. This $\delta^{15}N$ is a product of the combined balance of contributing sources and any subsequent fractionations undergone by these compounds in the sediments. The main original sources of N to the sediments in the Hawkesbury-Nepean River are agricultural run-off and effluent from STPs (NSW EPA, 1993). As is the case, sediment N would be expected to have $\delta^{15}N$ lying somewhere in between the $\delta^{15}N$ values of each of these sources if both are significant contributors. Fractionation during sediment processes is also significant, with most of the processes occurring enriching the sediments in $^{15}N$ (Mariotti *et al.*, 1981; Cifuentes *et al.*, 1988).

There was some difference between sites, with sediments at Boundary Creek being consistently higher in $\delta^{15}N$ than the other sites (Figure 4.10). This is probably due to an increased contribution of STP-derived N to the sediments at this site, as it is close to the Penrith STP. Effluent from this STP was found to have a mean $\delta^{15}N$ of 15.1 ± 0.9‰ (Section 4.3.3), and would be expected to increase the $\delta^{15}N$ of the sediment if it was a significant contributor to its overall N load. Differences between sites were greater for this experiment than for the STP and agricultural run-off experiments, and were large enough to almost overlap the characteristic values for each of those sources. Temporal variance over time was smaller than spatial variance, indicating that differences in the composition of sediment was related to site rather than variation through time.

Corrections were not made for the influence of fractionation by *M. umbilicatum* or the effect of nutrient concentration on the level of discrimination in the results presented in Section 4.5.3. Nitrate concentrations in the tank experiments were measured using a commercial aquarium nitrate kit, and were found to be consistently above 5mg.$L^{-1}$ in treatments

containing pore water from the Wilberforce Reach sediment. Pore water from sediment cores from other sites had a $NO_3^-$ concentration < 5mg.$L^{-1}$. The $\delta^{15}N$ of the sediment N from the Wilberforce Reach cores was therefore adjusted by –2.65‰ to allow for fractionation. This makes the mean for these cores 12.9‰ rather than 10.2‰ and adjusts the overall mean sediment $\delta^{15}N$ to 10.0‰

The $\delta^{15}N$ of sediment N from the three sites characterised fell within the ranges determined for sediment N in other studies (Pennock, 1987; Cifuentes *et al*., 1988; Mulholland and Olsen, 1991). The mean $\delta^{15}N$ of sediment N was also very different from the mean $\delta^{15}N$ of STP effluent and agricultural run-off (Sections 4.3.3 and 4.5.3 respectively). This is despite the closeness of some values to the lower end of STP effluent $\delta^{15}N$ and the higher end of agricultural run-off $\delta^{15}N$ due to the large range of $\delta^{15}N$ values found for sediment.

The aim of this experiment was not to distinguish accurately the $\delta^{15}N$ of sediment N, or to determine the processes occurring in the sediment. Instead, this experiment tried to identify a "characteristic sediment $\delta^{15}N$" in order to rule out the possibility that the $\delta^{15}N$ values identified in the field were due to enriched N being recycled to the water column from the sediments. Although some values came close to overlap with both STP effluent and agricultural run-off values, variance in the $\delta^{15}N$ of sediment between sites sampled was not large enough to prevent characterisation (Figure 4.10). Over the three times and four sites sampled, and allowing for fractionation, the mean $\delta^{15}N$ of sediment N was found to be 10.0‰. This mean value of was used as a "characteristic sediment $\delta^{15}N$" when interpreting the field results in Chapter 5.

## 4.5 Implications for field experiments

If characteristic and distinct $\delta^{15}N$ values of any possible N sources can be determined, it is possible to utilise $\delta^{15}N$ values as a "tracer" of N source. Each of the main N sources in the Hawkesbury-Nepean River, STP effluent, agricultural run-off, and N recycled from the sediments, were found to have distinct $\delta^{15}N$ values. Together with the size and direction of fractionations between source and *M. umbilicatum* determined in the experiments in Chapter 3, this makes it possible to determine the main sources of N contributing to the Hawkesbury-Nepean River from field experiments using the algae as a bioindicator.

The experiments described in this Chapter were undertaken to determine a characteristic $\delta^{15}N$ value for each of the main sources of N in the Hawkesbury-Nepean River. Characteristic $\delta^{15}N$ values could be obtained for each of the 3 sources. The $\delta^{15}N$ values for STP effluent, agricultural run-off and sediment were all found to be distinct from each other (Figure 4.11).

Effluent from STPs discharging into the Hawkesbury-Nepean River were found to have a characteristically high $\delta^{15}N$ due to the processes within the plant, with a mean of 15.6 ± 1.6‰ (Figure 4.8). Although variability in the $\delta^{15}N$ of effluent between STPs, which was probably due to slight differences in the treatment of wastewater at each STP, a discrete "STP effluent $\delta^{15}N$" could be characterised. Run-off from agricultural land into the Hawkesbury-Nepean River was found to have a much lower $\delta^{15}N$ than STP effluent, with a mean of was 4.1 ± 1.1‰ (Figure 4.9). The much lower $\delta^{15}N$ of this N source is the result of the combined animal wastes and inorganic fertiliser from the intensive horticulture and animal production in areas in the catchment, as well as any subsequent fractionations undergone by these compounds before entering the river. Despite the differences in $\delta^{15}N$ between sites, most likely due to the different composition of agricultural run-off in each area, an "agricultural run-off $\delta^{15}N$" could be effectively characterised.

Nitrogen recycled from sediments in the Hawkesbury-Nepean River was found to have a mean $\delta^{15}N$ of 10.0 ± 3.5‰ (Figure 4.10). This $\delta^{15}N$ is a product of the combined balance of contributing N sources and any subsequent fractionations undergone by these compounds in the sediments. As for the STP effluents and agricultural run-off sources, there was considerable variability in the $\delta^{15}N$ values for sediment between sites. This is probably due to differences in the relative contributions of STP and agricultural run-off-derived N to the sediments at each site. Differences between sites were however greater for this experiment than for the STP and agricultural run-off experiments. The range of individual sediment $\delta^{15}N$ values obtained overlapped the range of STP and agricultural run-off values. However, the mean $\delta^{15}N$ of all sediments sampled was distinct from the mean $\delta^{15}N$ values of STP effluent and of agricultural run-off (Figure 4.11). It was therefore possible to distinguish between the $\delta^{15}N$ of each of these 3 sources.

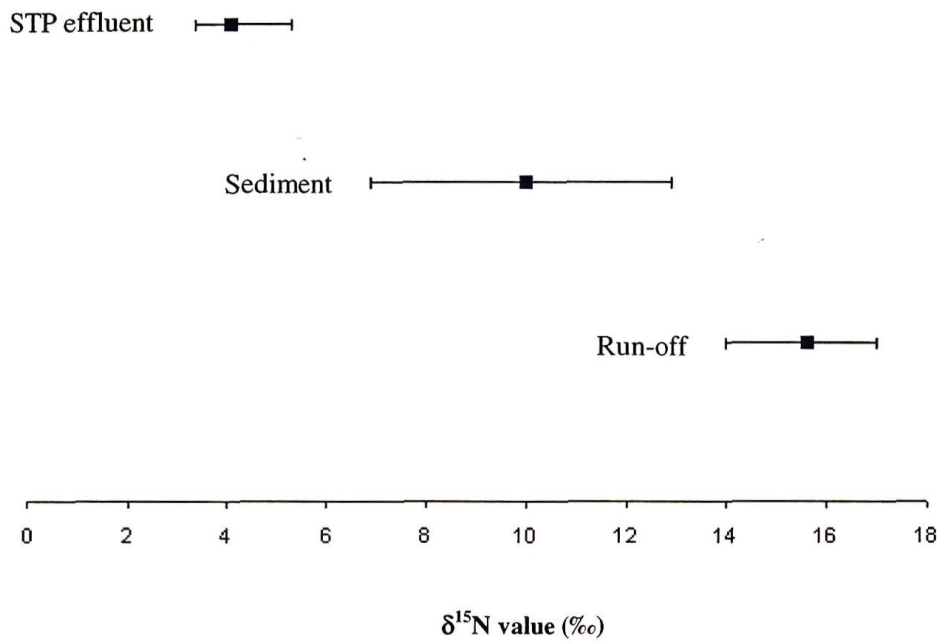



**Figure 4.11**: The range of $\delta^{15}N$ values for each of the main sources of N in the Hawkesbury-Nepean River. Each source is distinct.

The results of these experiments are used in the following Chapter to interpret the final $\delta^{15}N$ values of algae deployed in the field in terms of N source.

# CHAPTER 5: THE MAIN SOURCES OF NITROGEN CONTRIBUTING TO THE EUTROPHICATION OF THE HAWKESBURY-NEPEAN RIVER.

## 5.1 Introduction

### *5.1.1 Experimental design*

Assessing impacts such as pollution in aquatic ecosystems is subject to many limitations and must be measured using a logical experimental structure. In this study, the impact of each of a number of potential N sources to the overall eutrophication of a river system is to be determined. In all studies of this type, the accuracy and precision afforded by the sampling design must be considered as well as the biological and logical aspects (Figure 5.1).

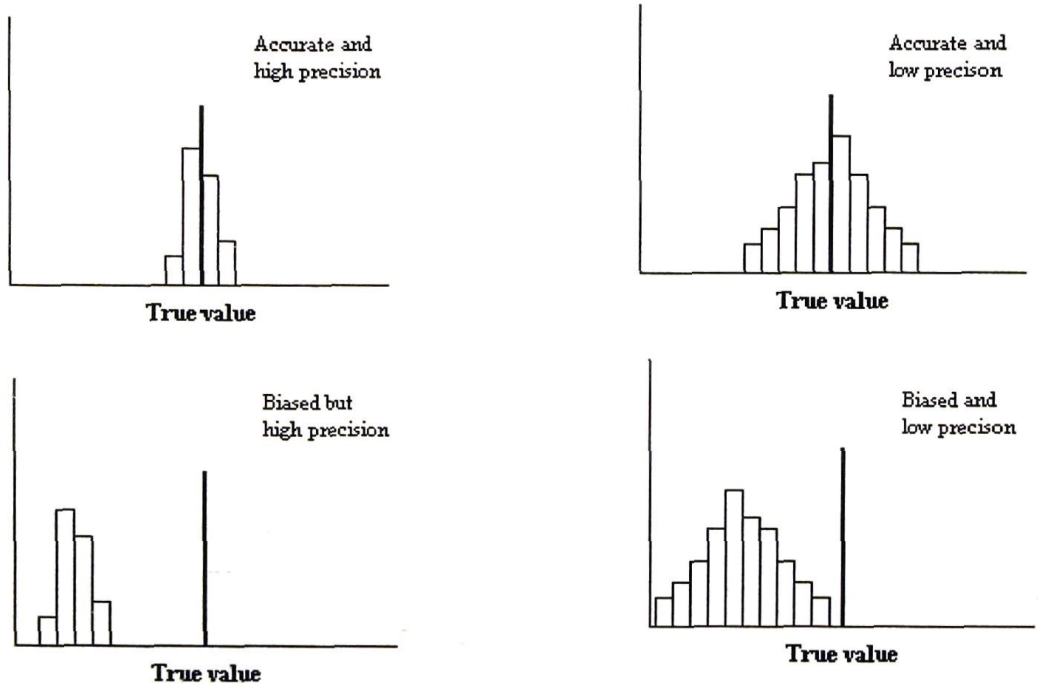



**Figure 5.1**:Accuracy and precision of sampling (adapted from Kingsford & Battershill, 1998).

Accuracy refers to the extent to which the mean of a sample departs from the "real" or true population mean. Precision refers to the size of the variation of the data around the mean, and is usually expressed as the ratio of standard error to the mean (Figure 5.1; Kingsford and Battershill, 1998). The accuracy and precision of the samples in this study is discussed in Section 5.5.

Manipulative experiments are extremely useful in environmental studies and are usually the only way to distinguish unequivocally between competing models (Underwood, 1997). To successfully utilise manipulative experiments, they must be carefully designed to prevent artefacts or confounding factors and to incorporate proper controls. The approach to the collection and interpretation of $\delta^{15}N$ data using a standardised measuring device sets this study apart from other field studies utilising $\delta^{15}N$ values to determine N source (see Chapter 2 for a full discussion of this issue).

Once a problem is expressed as a hypothesis or a series of linked hypotheses, decisions need to be made as to the number of replicates, how sampling effort should be allocated and how the data should be treated once it is collected (Kingsford and Battershill, 1998). Decisions on the allocation of the sampling effort lead to the design of the experiment. Nested sampling designs are often utilised in impact studies and are a powerful approach for dealing with temporal and spatial variability on a number of scales. A nested design was utilised for the field experiments in this study.

An important decision in the design of these types of experiments is the allocation of fixed and random factors. Fixed factors are defined as being those where all possible treatments relevant to the hypothesis are sampled randomly. Random factors are where treatments are chosen as representative of a much larger set of possibilities (Equation 6; Kingsford and Battershill, 1998).

The allocation of fixed and random factors in this study is discussed in section 5.3.2.

The number of degrees of freedom (df) is also an important consideration in the design of an experiment, and should be large for the factors of greatest interest in order to increase the likelihood of detecting any real differences due to the factors under investigation. Degrees of freedom dictate F-values, which are very high at low degrees of freedom and drop rapidly as df increase (Berry and Lindgren, 1990). Low degrees of freedom therefore also greatly increase the chance of Type II error (Table 5.1; Underwood, 1997; Kingsford and Battershill, 1998). Degrees of freedom for all factors in this study are outlined in Table 5.1.

**Table 5.1**: Type I and II errors in the interpretation of experiments (adapted from Underwood, 1997).

| **Outcome of statistical test is to:** | **Null hypothesis ($H_o$) is really:** | |
|---|---|---|
| | ***True*** | ***False*** |
| ***Reject $H_o$*** | Type I error occurs with a probability $\alpha$ (set by the experimenter) | Correct conclusion: false $H_o$ is rejected |
| ***Retain $H_o$*** | Correct conclusion: true $H_o$ is retained | Type II error occurs with probability $\beta$ |

P-values (and hence $\alpha$) are usually set by convention as P = 0.05, yet there are arguments that this is not always the most appropriate value, particularly for impact studies. It is frequently suggested that P-values should be set on a case-by-case basis, with the possible environmental cost of a Type II error being taken into account when setting the P-value for a particular study. For this study, P was initially set at P=0.05 (see Sections 5.3.2 and 5.4.3 for a full discussion of the P values set for this study).

Experimental design and analysis choices are discussed in detail for the field experiments in Section 5.3.2.

### *5.1.2 Determining sources of nitrogen in the Hawkesbury-Nepean River*

Eutrophication has been an issue in the Hawkesbury-Nepean River since the 1970s. It contributes to a number of problems in the river such as algal blooms, oxygen depletion and disruption of sediment processes (Section 1.1). In order to properly manage this problem, it is vital to be able to identify and quantify the relative contributions of nutrient sources to the river. The work described in this thesis deals specifically with identifying sources of nitrogen.

Both diffuse and point sources contribute to the overall eutrophication of the Hawkesbury-Nepean River. Effluent from sewage treatment plants has been identified as a major source of N, despite a denitrification step being introduced to many STPs in recent years (Section1.3.2). Diffuse run-off from agricultural land is also a significant contributor to the N load of the river (Sections 1.3.3 and 4.4.1). Run-off from agricultural land and effluent from STPs are virtually identical in their chemical composition, making them indistinguishable by traditional chemical methods once in the water column. However, the $\delta^{15}N$ of each source is distinct, and provides a method of identifying the relative contributions of each to eutrophication.

However, there are a number of limitations to the use of $\delta^{15}N$ values as a "tracer" of N source in aquatic ecosystems. These are discussed in detail in Chapter 2. The use of *M. umbilicatum* as a well-characterised "bioindicator" to collect $\delta^{15}N$ data eliminates many of these problems, and also provides a mechanism with which to estimate any fractionations between source and sink (Section 2.5).

Environmental factors may also influence the final $\delta^{15}N$ value of an alga. Of the environmental factors investigated, light levels and nitrate concentration were found to have a significant effect on the level on fractionation occurring between source N and *M. umbilcatum* (Chapter 3). Environmental data were collected at each of the sites algae were deployed in the field and final $\delta^{15}N$ values of *M. umbilicatum* were corrected for the effect of fractionation caused by light or nitrate levels over determined threshold values (Section 5.3.2).

In order to use $\delta^{15}N$ values as a "tracer", it is also essential to characterise the $\delta^{15}N$ values of any possible N sources. For the Hawkesbury-Nepean River, STP effluent and agricultural run-off were isolated and a $\delta^{15}N$ profile of each was established using *M. umbilicatum* (Chapter 4). A characteristic $\delta^{15}N$ for N recycled from the sediments was also determined. Each of these sources was found to have a distinct $\delta^{15}N$ (Section 4.4).

### *5.1.3 Aims*

The overall aim of this study reported in this Chapter was to determine the relative contribution of each of the various N sources entering the Hawkesbury-Nepean River and contributing to its eutrophication. This allows for the relative impact of each source upon the river ecosystem to be assessed.

Using *M. umbilicatum* as a "bioindicator" to collect $\delta^{15}N$ data from the Hawkesbury-Nepean, and estimating fractionation under various conditions has greatly reduced experimental error in this study (Chapter 3). The use of *M. umbilicatum* also allows source $\delta^{15}N$ and identity to be clearly established (Chapter 4).

The aim of the experimental design presented in this Chapter was to collect $\delta^{15}N$ data in such a way as to allow the relative contribution of agricultural run-off and STP effluent to the eutrophication of the Hawkesbury-Nepean River to be established. The appropriate adjustments to the $\delta^{15}N$ values of the *M. umbilicatum* algae as determined by the experiments outlined in Chapters 3 and 4 were also carried out.

$\delta^{15}N$ data were collected using *M. umbilicatum* along the length of the Hawkesbury-Nepean River for a period of 3 years. The main sources of N found to be contributing to the eutrophication of the Hawkesbury-Nepean River are identified and discussed in this Chapter.

## 5.2 Pilot study

### *5.2.1 Introduction*

A pilot study was carried out in April 1997 to test the range of $\delta^{15}N$ values in plants growing in the Hawkesbury-Nepean River and identify suitable access points and sites for the field study.

*Microdictyon umbilicatum* algae and apparatuses were not used for the pilot study, as the aim of this exercise was a rough estimate of $\delta^{15}N$ ranges only and M. umbilicatum had not yet been decided upon as the bioindicator organism. Instead, samples from the leaves or thalluses of endemic plants were collected at each site (Figure 5.2). It was problematic to find suitable samples at each site, and some planned sites were not sampled due to a lack of appropriate plant species. Due to the wide range of salinities and other environmental conditions encountered along the length of the Hawkesbury-Nepean River, it was not possible to sample the same species of plant at each site. Plant species sampled at each site are indicated in Table 5.2. These samples were intended only to give an estimate of the range and pattern of $\delta^{15}N$ values found in the Hawkesbury-Nepean River and were not repeated due to the many problems associated with sampling endemic plants for $\delta^{15}N$ values (outlined in detail in Chapter 2).

### 5.2.2 Materials and Methods

Four individual plants, at a distance of at least 10m from each other within a site, were sampled at each of the sites selected for the pilot study (Figure 5.2). The species of plant sampled varied between sites due to availability and is indicated in Table 5.2. .

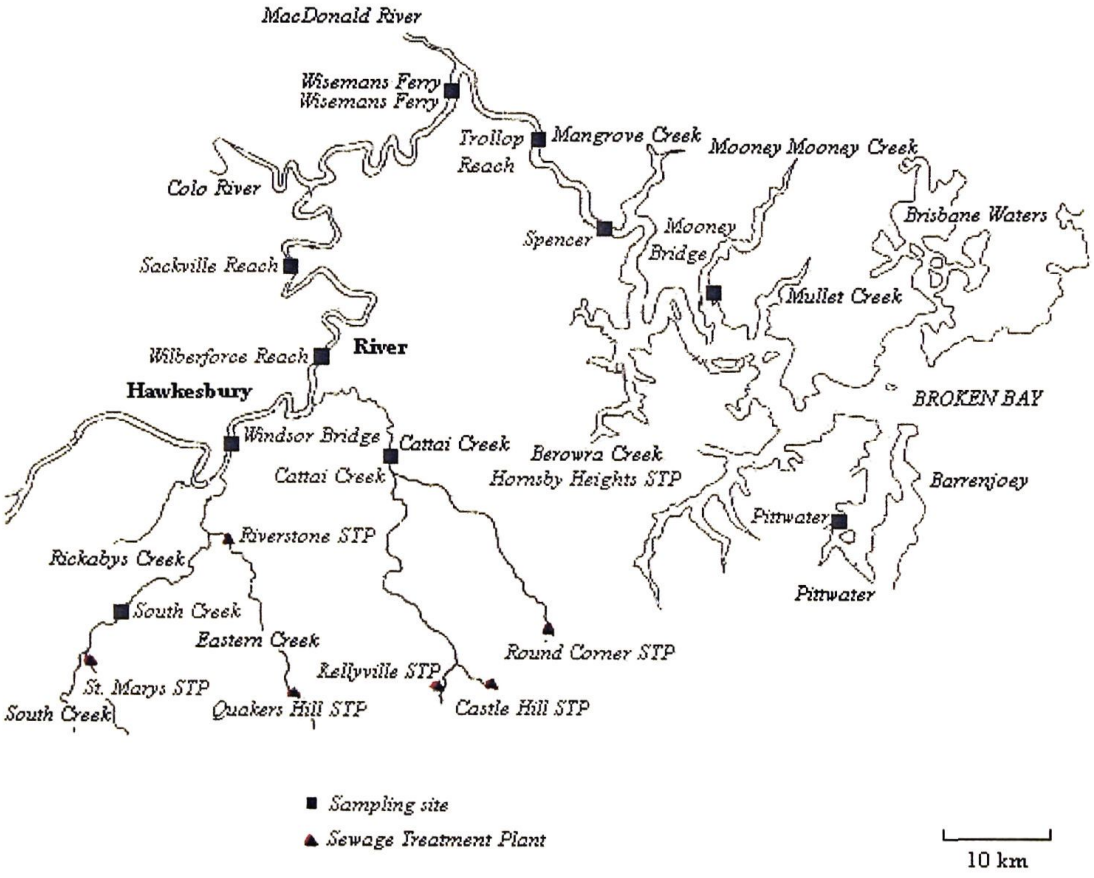



**Figure 5.2**: Sampling sites in the Hawkesbury-Nepean River for the collection of plant species for the pilot study.

**Table 5.2**: Plant species sampled at each site in the Hawkesbury-Nepean River for the pilot study carried out in April 1997.

| **Sample site** | **Plant species sampled** |
|---|---|
| South Creek | *Vallisneria* spp. |
| Cattai Creek | *Vallisneria* spp. |
| Windsor Bridge | *Egeria* spp. |
| Wilberforce Reach | *Egeria* spp. and green filamentous algae spp. |
| Sackville Reach | *Egeria* spp. |
| Wisemans Ferry | *Juncus* spp. |
| Trollop Reach | *Egeria* spp. |
| Spencer | *Juncus* spp. |
| Mooney Bridge | Kelp spp. |
| Pittwater | Kelp spp. |

After collection, plants were rinsed in clean water from the laboratory (with the same approximate salinity as the field site). Plants were stored in individual 500mL polyurethane jars containing clean water for transport back to the laboratory. In the laboratory, plants were rinsed again in distilled water and any epiphytes or unhealthy sections of the plant were removed. Samples were then prepared for analysis as outlined in Section 3.2.2.

### *5.2.3 Results*

Plants of all species sampled from the Hawkesbury-Nepean River had mid-range to high $\delta^{15}N$ values (Figure 5.3). Plants sampled from locations closer to STP outfalls were higher in $\delta^{15}N$ than plants sampled from locations further from STP outfalls (see Figure 5.2).

There was no obvious pattern in the differences in the $\delta^{15}N$ due to plant species (Figure 5.3). However, this was confounded by site in the design of the experiment, with, for example, *Vallisneria* species able to be sampled only at South and Cattai Creeks, both of which are close to STP outfalls (Figure 5.2).

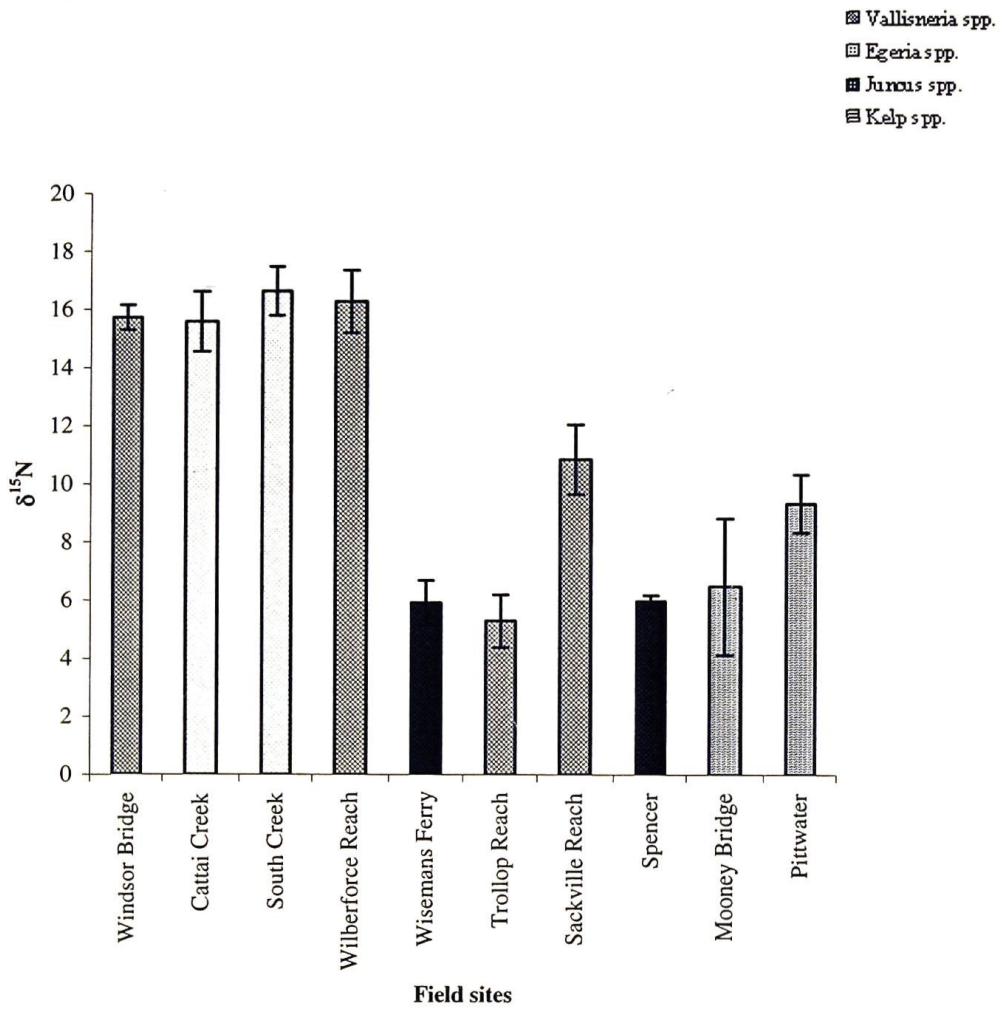



**Figure 5.3**: Mean $\delta^{15}N$ in various species of plants collected from the Hawkesbury-Nepean River (values are mean $\delta^{15}N \pm SD$ (‰) from the 4 replicate plants sampled at each location).

### *5.2.4 Discussion*

Different species of plants may assimilate N in very different ways. This in turn will affect the amount of fractionation and the final $\delta^{15}N$ of the plant tissue. Using many different plant species to collect $\delta^{15}N$ signals from the river would introduce a number of confounding factors, including differences in fractionation due to species, differences in responses to environmental conditions and difficulties in consistently selecting the same plant organs for analysis. In plant-based studies, vegetation type, species and plant functional type can all affect $\delta^{15}N$ signals, yet are often ignored when interpreting data (Handley and Scrimgeour, 1997).

Significant differences due to genetic variation can exist between species (even those from the same order) in the extent to which N is fractionated during uptake and assimilation. This can lead to differences in the $\delta^{15}N$ values of tissues due to biochemical variation between individuals and not to other, external, factors. Genetic variability between individuals, particularly in wild populations, may also result in differences in the enzymatic pathways used to uptake and metabolise N compounds.

The selection of sample material from an organism is extremely important, as each organ may assimilate, fractionate or store N in a different manner (Ledgard *et al.*, 1985; Yoneyama, 1995; Evans *et al.*, 1996; Handley and Scrimgeour, 1997). This can lead to marked differences in $\delta^{15}N$ between organs. In the pilot study, consistency between plant organs sampled was not possible due to the wide variation between species morphology, and this certainly increased variability in the final $\delta^{15}N$ values. Sources of $\delta^{15}N$ variation in plants are discussed in detail in Chapter 2.

Because of the potential difficulties associated with using different wild plant species outlined above, it was decided to develop a bioindicator plant that could be cultured in the laboratory and then deployed in field trials. Using *M. umbilicatum* as a bioindicator to collect $\delta^{15}N$ values from the river would eliminate many of the sources of variation outlined above and this alga was used in further experiments. Increases in the precision of the field data due to the use of the bioindicator are discussed in Sections 5.4.2 and 5.5.

## 5.3 General experimental methods

*Microdictyon umbilicatum* algae were cultured as described in Section 3.2.1 for these experiments. However, the salinity of the growth medium was adjusted to approximately match that of the intended field site of each group of algae (see Section 3.2.5 for details on salinity adjustment methods).

Half the *M. umbilicatum* algae were cultured in high $\delta^{15}N$ nutrient, and half in low $\delta^{15}N$ nutrient to provide a control for the turnover of N during the experiments (Section 3.2.1). High and low $\delta^{15}N$ algae were allocated randomly, 3 of each per apparatus. Turnover results for these experiments are presented in Section 5.3.2, but were not performed for all replicate experiments due to the reliable turnover of N in *M. umbilicatum* within the 14 day experimental period and the considerable cost of analysing each replicate alga.

Algae were deployed in the river in custom-made apparatuses designed to allow them contact with the water column but prevent them from being washed away (Section 4.2.1, Figure 4.1).

In the field, algae were transferred from 500mL polyurethane jars containing their growth medium directly to the apparatuses. Three algae from high $\delta^{15}N$ growth media and 3 from low $\delta^{15}N$ growth media, where applicable, were randomly allocated to each apparatus. For replicates where turnover was measured, a total of 12 replicate algae were deployed at each site at each time, 6 per apparatus. For replicates where turnover was not measured, 3 algae were allocated to each apparatus, for a total of 6 replicates per site (see Figure 5.4 for experimental design). Apparatuses were < 50m apart. Results from both apparatuses were pooled for the graphs presented in Section 5.4.3. Algae were left in the field for 14 days before collection.

After collection, algae were immediately removed from the apparatus and rinsed in clean water from the laboratory (with the same approximate salinity as the field site). Algae were stored in individual 500mL polyurethane jars containing clean water for transport back to the laboratory. In the laboratory, algae were rinsed again in distilled water and any epiphytes or unhealthy sections of the algae were removed. Samples were then prepared for analysis as described in Section 3.2.2.

Alga biomass was recorded as wet volume at the beginning and end of the two-week period, and replicates were discarded if their biomass had not increased by at least 50%.

In order to define a characteristic $\delta^{15}N$ for each site in the Hawkesbury-Nepean River, algaewere deployed in the river at sites ranging from Penrith Weir to Broken Bay (Figure 5.3).

Ten replicate field experiments were carried out in August 1997, December 1997, May 1998, August 1998, October 1998, November 1998, March 1999, May 1999, June 1999 and August 1999. Turnover data were analysed for the August 1997, December 1997, May 1998 and August 1998 replicates.

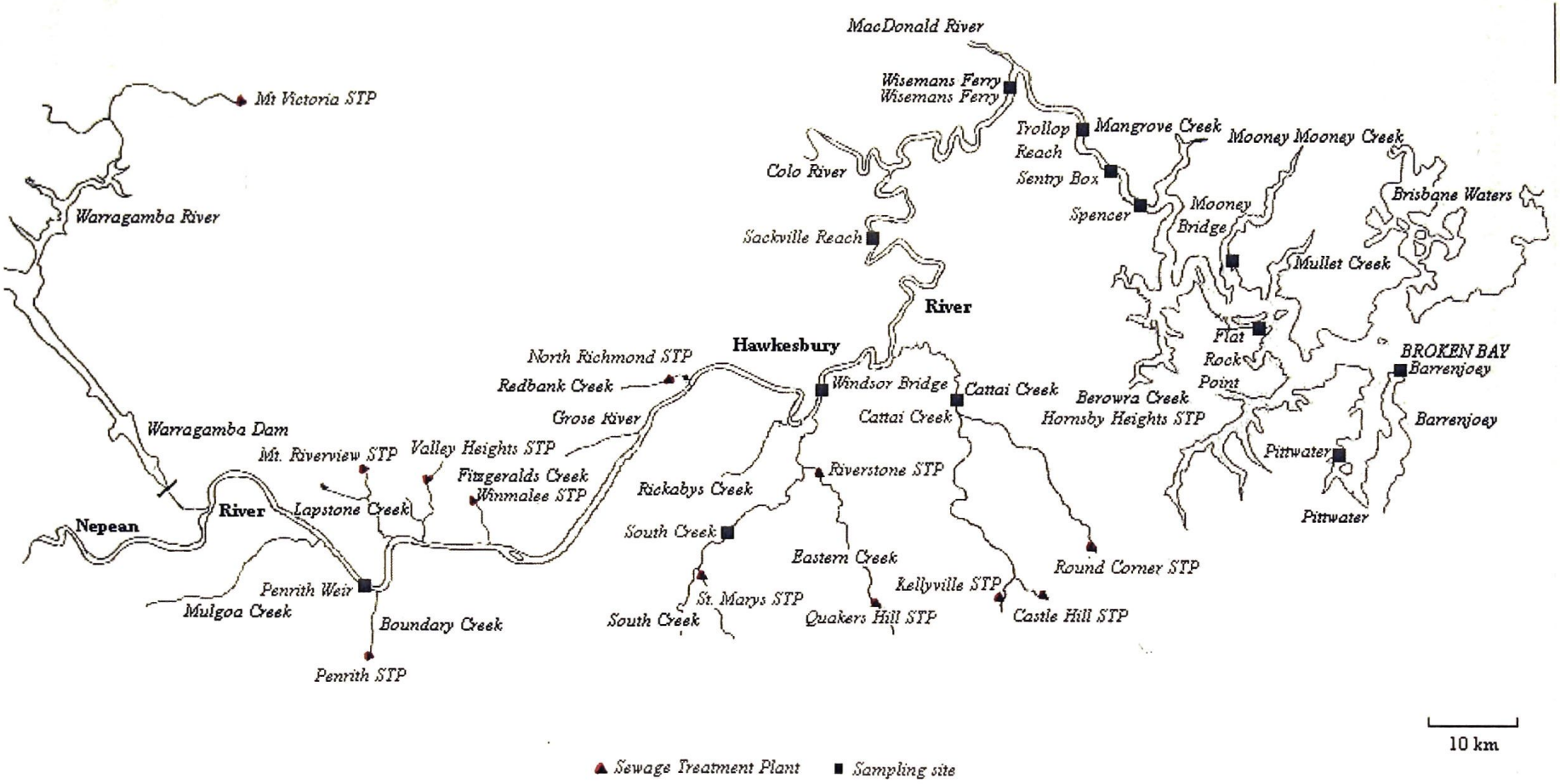



**Figure 5.4**: Sampling sites in the Hawkesbury-Nepean River for the collection of $\delta^{15}N$ data by *Microdictyon umbilicatum*.

### *5.3.1 Experimental design and statistical tests*

*Experimental design*

A partially hierachical design was used to investigate the effect of N sources on the $\delta^{15}N$ of *M. umbilicatum* bioindicator algae in the Hawkesbury-Nepean River (Figure 5.5). The difference in the turnover of N controls (the "high" and "low" algae) were found to be non-significant in the analysis and are therefore discussed separately (Section 5.4.1).

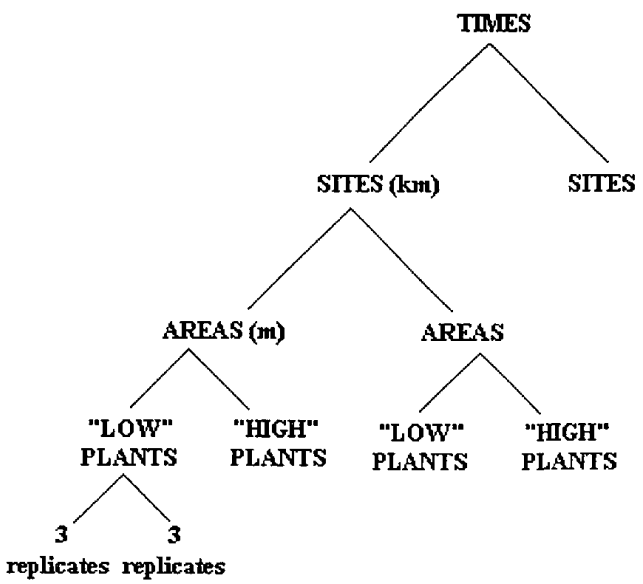



**Figure 5.5**: General design of experiments investigating $\delta^{15}N$ of *Microdictyon umbilictatum* at various sites in the Hawkesbury-Nepean River.

Each of the factors in this experiment was random. Times (sampling months) were randomly allocated in the 3 year sampling period, and sites were allocated approximately evenly along the rivers length. Areas were randomly nested within sites, and replicated algae randomly allocated to each apparatus. This greatly increased the power of the statistical tests performed.

*Corrections to $\delta^{15}N$ to allow for the effect of environmental conditions*

Corrections to the final $\delta^{15}N$ of the *M. umbilicatum* bioindicator algae were done to allow for the effect of high nutrient concentrations (+ 3.0 ‰) or low light (+ 2.7‰). Results were corrected when environmental conditions were above the threshold values determined in Chapter 3 for a significant part of the experimental period (determined as ≥ 10 out of the 14 days). Environmental conditions were measured in the field on days 1 and 14 and events such as flooding in the interim period were also noted.

If both low light and high nutrient conditions were encountered by *M. umbilcatum* at a site, these effects were considered to be additive, and the combined fractionation of 5.62‰ (Section 3.5) was added to the final $\delta^{15}N$ value of the algae from this site. This is further discussed in Section 6.2.1.

**Table 5.3:** *Microdictyon umbilicatum* $\delta^{15}N$ values adjusted for the effect of environmental conditions.

| Sampling time | Site | Environmental condition exceeding threshold value |
|---|---|---|
| August 1997 | South Creek | Nitrate |
| | Cattai Creek | Nitrate |
| | Sackville | Light |
| | Pittwater | Light |
| December 1997 | South Creek | Nitrate |
| | Cattai Creek | Nitrate |
| | Pittwater | Light |
| May 1998 | South Creek | Nitrate |
| | Cattai Creek | Nitrate |
| | Windsor Bridge | Nitrate |
| | Pittwater | Light |
| August 1998 | South Creek | Nitrate |
| | Cattai Creek | Nitrate |
| | Pittwater | Light |

The effect of rainfall on the $\delta^{15}N$ of the *M. umbilicatum* bioindicator algae was also monitored. It would be expected that the proportion of N in the river from agricultural run-off would greatly increase in the period immediately after rainfall, as this is when animal wastes and inorganic fertiliser are washed from the land into the river (Section 4.1.2). "Significant rainfall" was defined as rainfall which was consistent over 3 days or more, and raised water level of the river at Freemans Reach to above 1.25m for 2 days or more. These data were

collected by automatic recording equipment owned by Manly Hydraulics Laboratory, Sydney, Australia. This occurred immediately before the August 1997, May 1998, March 1999 and June 1999 sampling periods.

*Statistical tests*

Analysis of variance (ANOVA) was used to test for differences in the $\delta^{15}N$ of *M. umbilcatum* algae between times, sites or areas. Cochran's *C*-test was used prior to ANOVA to test for homogeneity of variances ($P = 0.05$) among sample groups. Transformations were done where necessary in order to meet ANOVA's assumptions of normality (Section 5.3).

*Cochran test*

$C = 0.6161$ $P < 0.01$

This value of C is significant, indicating hererogeneity of variances. ANOVA was therefore run with significance set at a lower level of $P < 0.01$ (Table 5.3). In all analyses, Cochran's *C*-test was significant ($P < 0.05$) even after transformation, indicating heterogeneity of variances.

Comparative tests such as ANOVA when used on large and balanced data sets are known to be relatively robust to heterogeneous variances (Box, 1953). This was the case with the data set in this set of experiments, so ANOVA was performed, but critical *P* values set at a lower level of $P < 0.01$ for significance (Underwood, 1997).

Student-Newman-Keuls (SNK) tests were used to compare means. In SNK tests, ">>" or "<<"indicate significance at $P < 0.01$, ">" or "<" indicate significance at $P < 0.05$, and "=" indicates $P > 0.05$ and is not significant (NS). ANOVA and SNK tests were done using GMAV5, Analysis of Variance Package (A. Underwood & G. Chapman, Institute of Marine Ecology, 1993).

Some replicates were lost due to missing apparatuses or alga death on a few occasions. In order to maintain a balanced design (necessary for the statistical package used), these values were replaced with the mean value for this replicate. Due to the very small standard deviation seen within each replicate set, this is unlikely to have affected the outcome of the statistical tests performed.

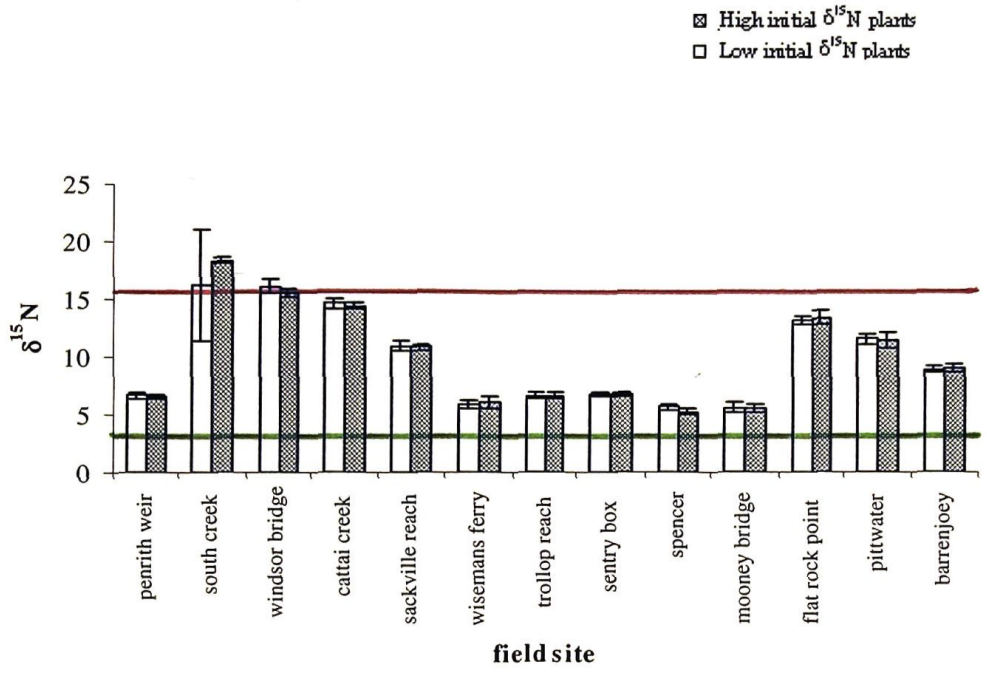
## 5.4 Results

### *5.4.1 Nitrogen turnover*

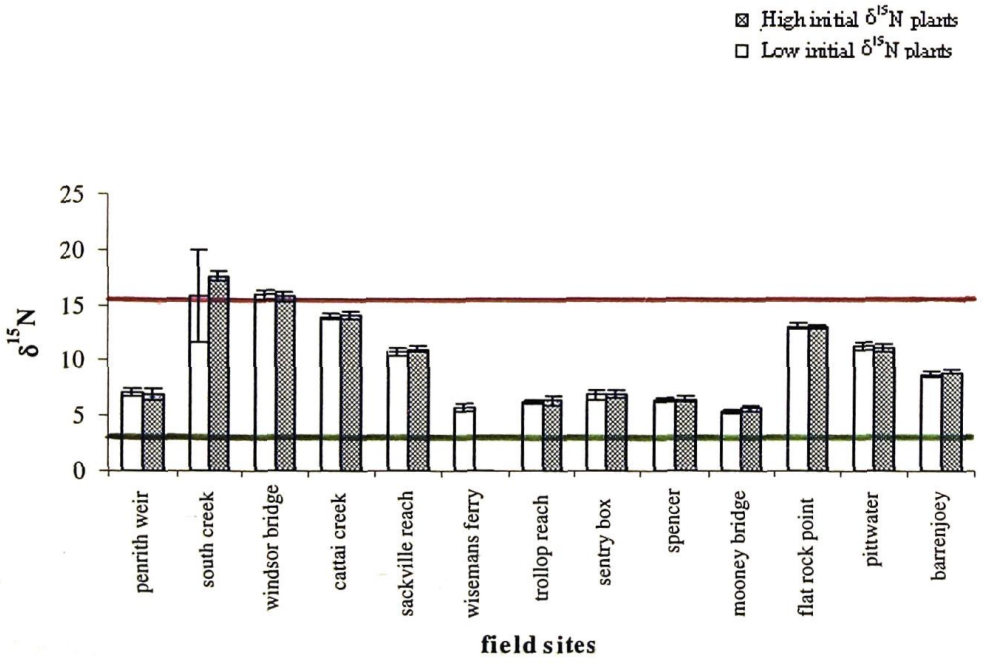
Nitrogen turnover in *M. umbilcatum* was consistently achieved within the 14-day experimental period (Figure 5.6). Algae from both "high" and "low" initial $\delta^{15}N$ regimes had equilibrated to the same $\delta^{15}N$ (reflecting that of the Hawkesbury-Nepean River) within 14 days. This pattern was consistent for all replicate experiments (Figure 5.6).

Nitrogen turnover in *M. umbilicatum* may not have been as rapid in the field as the laboratory, where N was turned over in the algae in 7-10 days (Figure 3.2). However, it was achieved within the 14-day experimental period in all cases where it was measured (Figure 5.6). This allowed for the "high" and "low" algae $\delta^{15}N$ values to be pooled in later experiments. This reduced the cost of the study considerably. It was decided to use algae grown in "low" initial $\delta^{15}N$ regimes in preference to those grown in a "high" initial $\delta^{15}N$ regime in order to ensure that any possible bias due to the growth environment would lower rather than raise $\delta^{15}N$ values. This is discussed further in Section 5.5.

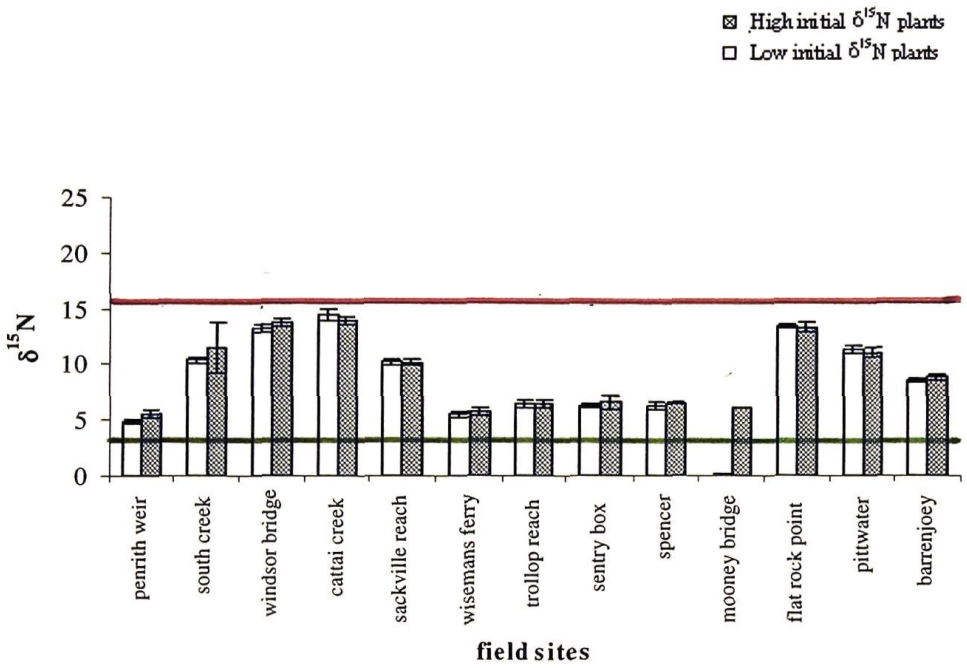
**A**: August 1997




**B**: December 1997



C: May 1998




D: August 1998

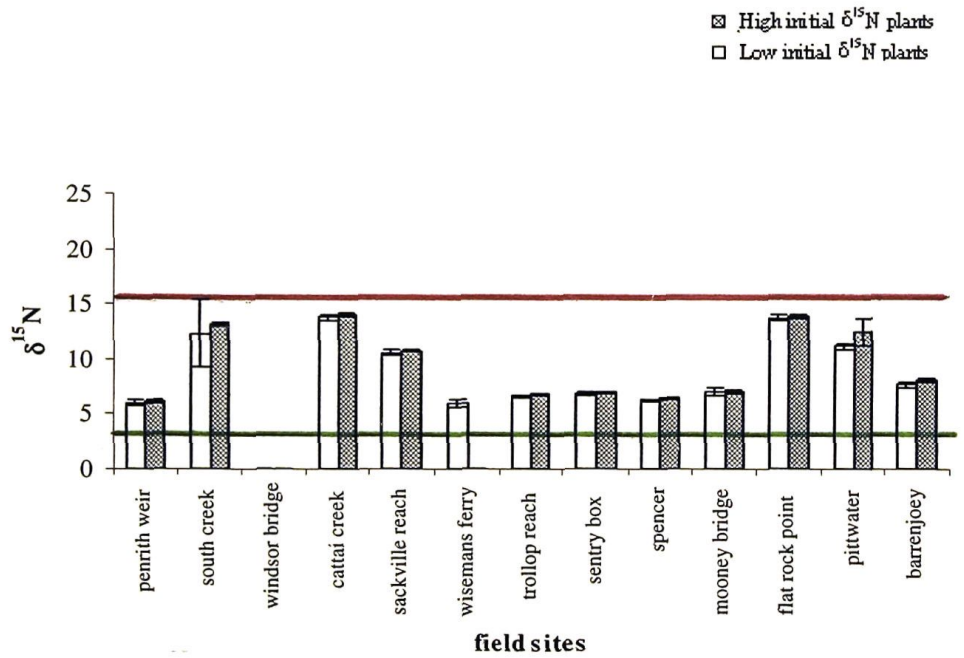


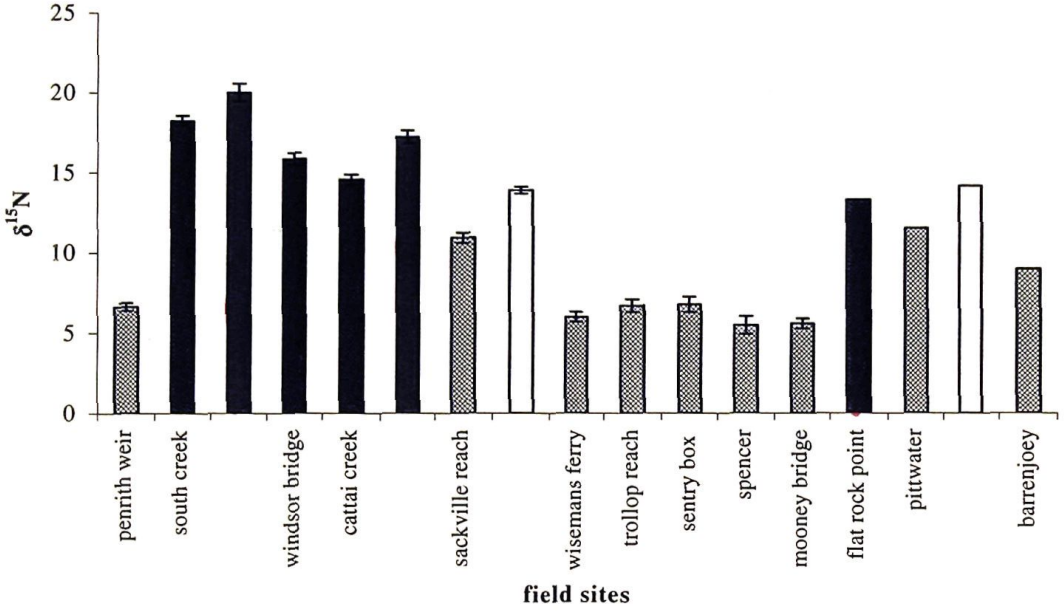

**Figure 5.6**: Mean $\delta^{15}N$ as measured by *Microdictyon umbilicatum* cultured in high and low $\delta^{15}N$ nutrient solution then deployed to sites in the Hawkesbury-Nepean River (values are mean $\delta^{15}N \pm SD$ (‰) from the 6 replicate algae deployed at each location). Red lines indicate mean $\delta^{15}N$ of STP outfalls and green lines indicate $\delta^{15}N$ of agricultural run-off.

### *5.4.2 Field $\delta^{15}N$ values*

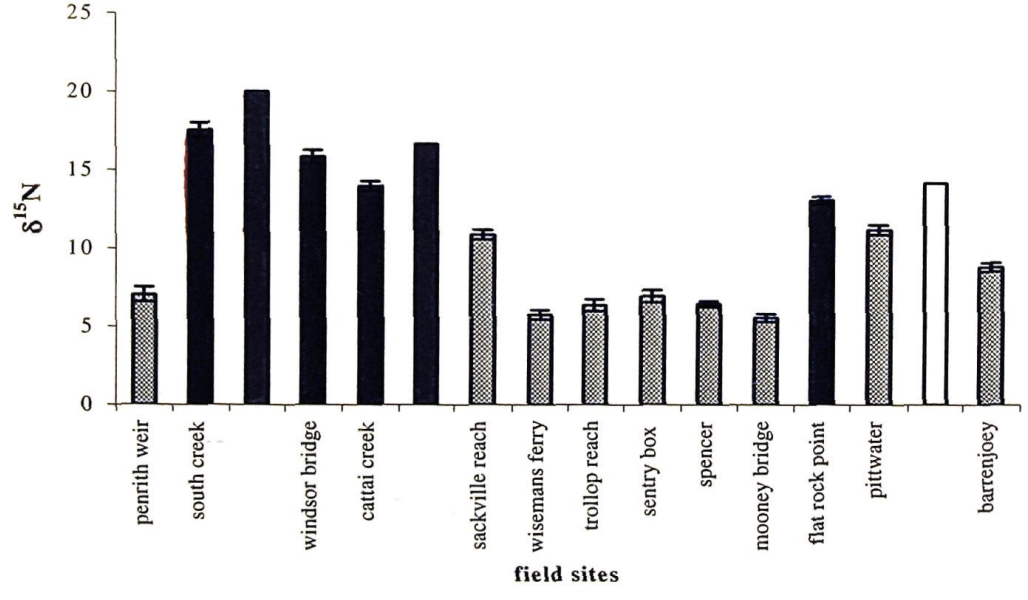
*Microdictyon umbilicatum* algae deployed to the Hawkesbury-Nepean River as bioindicators had mid-range to high $\delta^{15}N$ values at all times sampled (Figure 5.7). Algae sampled from locations closer to STP outfalls (South Creek, Windsor Bridge, Cattai Creek and Flat Rock Point) were consistently higher in $\delta^{15}N$ than algae sampled from locations further from STP outfalls (Figure 5.7). This pattern of elevation of $\delta^{15}N$ near STPs was extremely consistent between replicate experiments (Figure 5.7), even though sites further from STPs varied somewhat in their actual $\delta^{15}N$ values and relative rankings between times (see SNK test results below). Adjustments to the final $\delta^{15}N$ values of the *M. umbilicatum* bioindicators from some sites and times were made to allow for the influence of environmental conditions on the fractionation of $\delta^{15}N$. A full list of sites and times adjusted for the effect of environmental conditions is in Appendix 1. However, these adjustments left the overall pattern of $\delta^{15}N$ unaffected (Figure 5.7). Rainfall also had little effect on the pattern of very high $\delta^{15}N$ values near STP outfalls (Section 5.4.4).

The precision of the samples taken by the *M. umbilicatum* bioindicators (as measured by standard deviatation from the mean) is much less than for the measurements taken from a variety of plant species at the same sites in the pilot study (Figure 5.5). The precision and accuracy of samples from *M. umbilicatum* is discussed in Section 5.5.

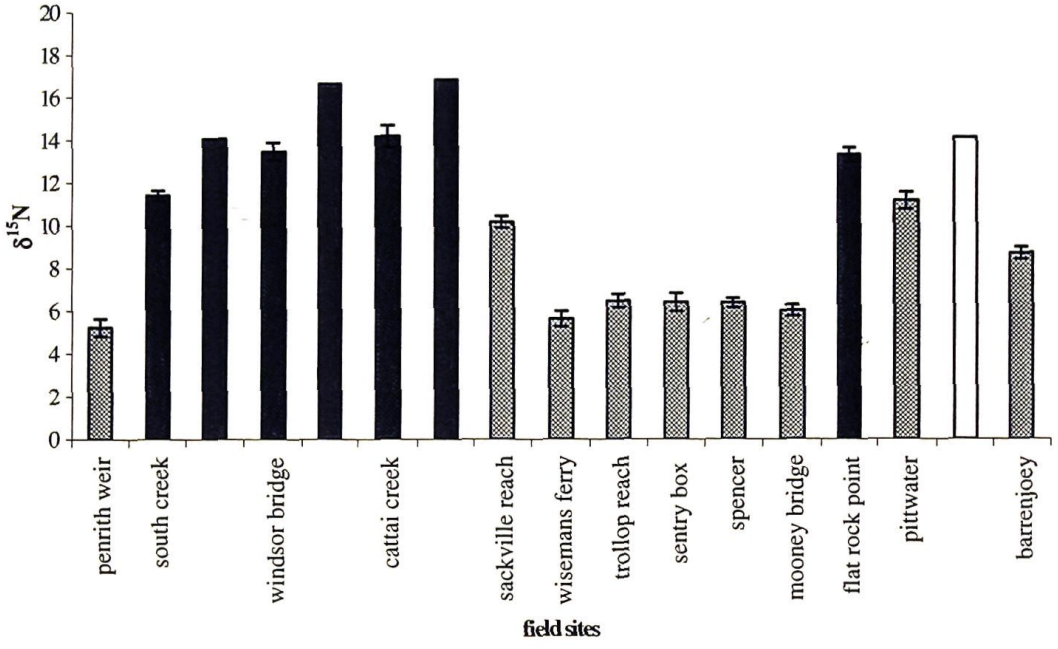
**A**. August 1997



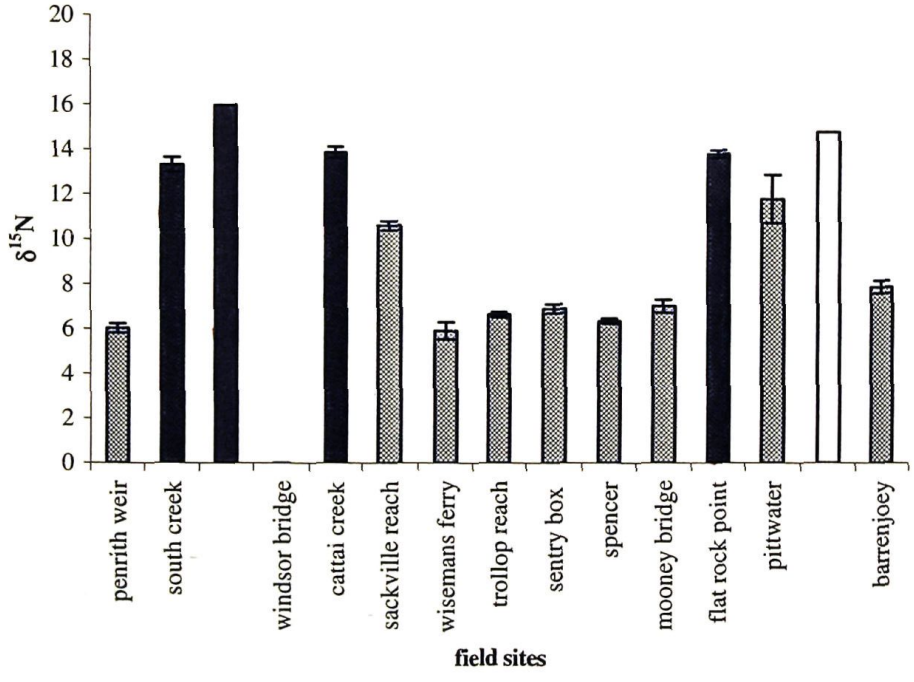

**B**. December 1997



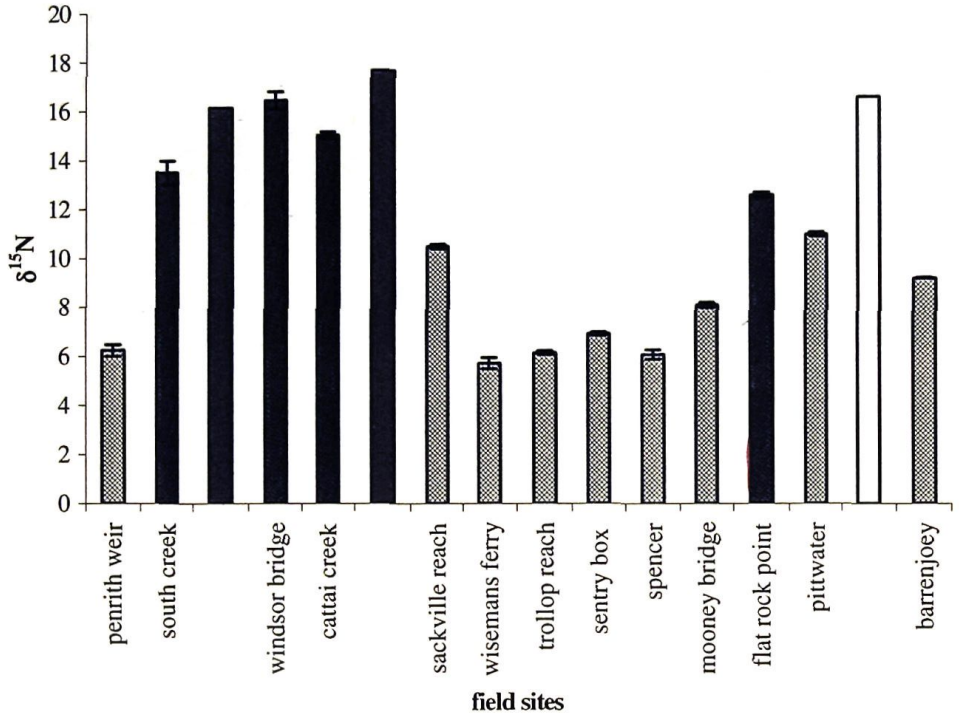
C. May 1998



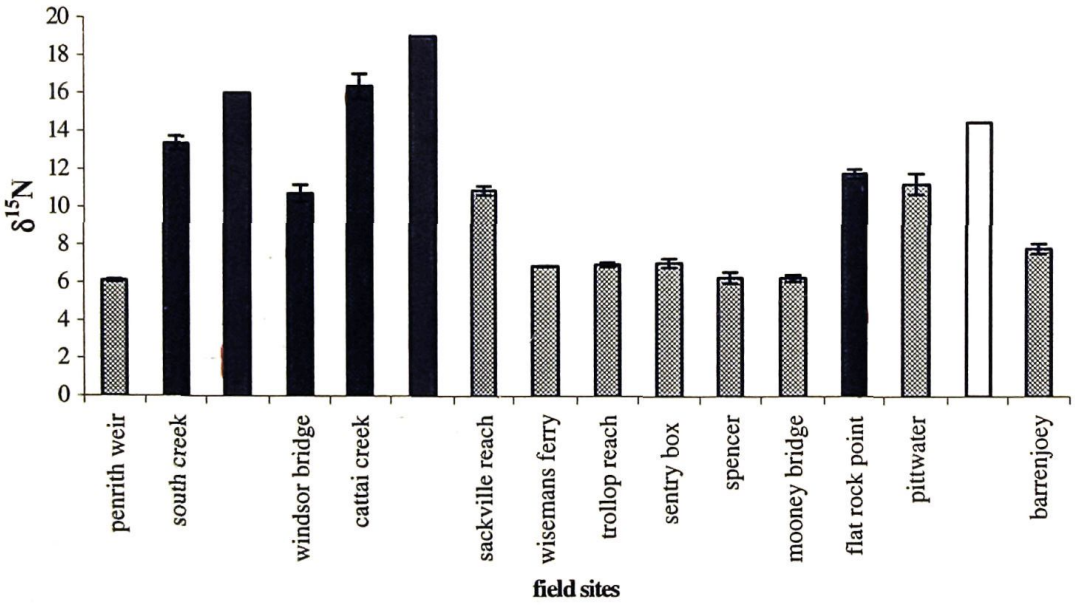

D. August 1998



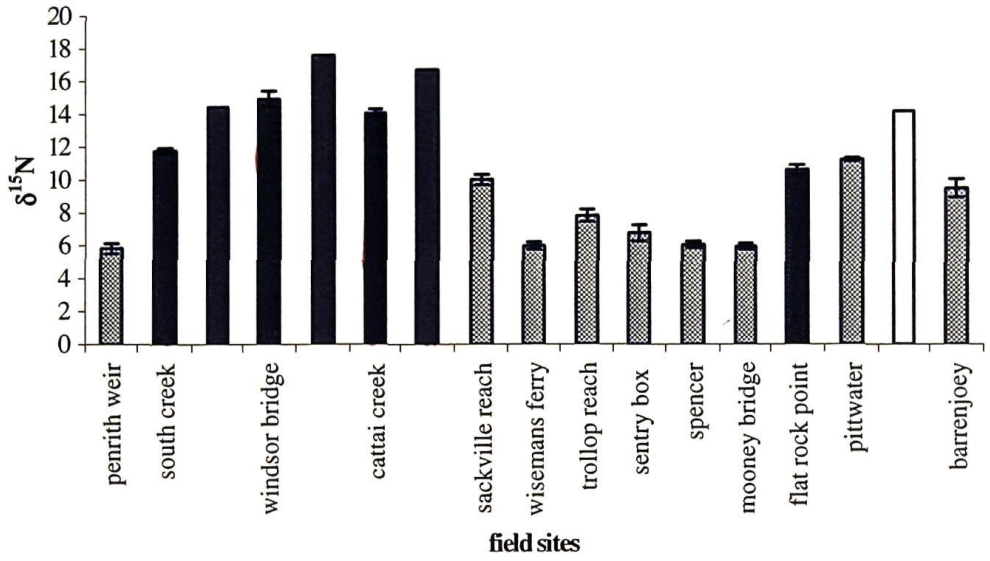
**E.** October 1998



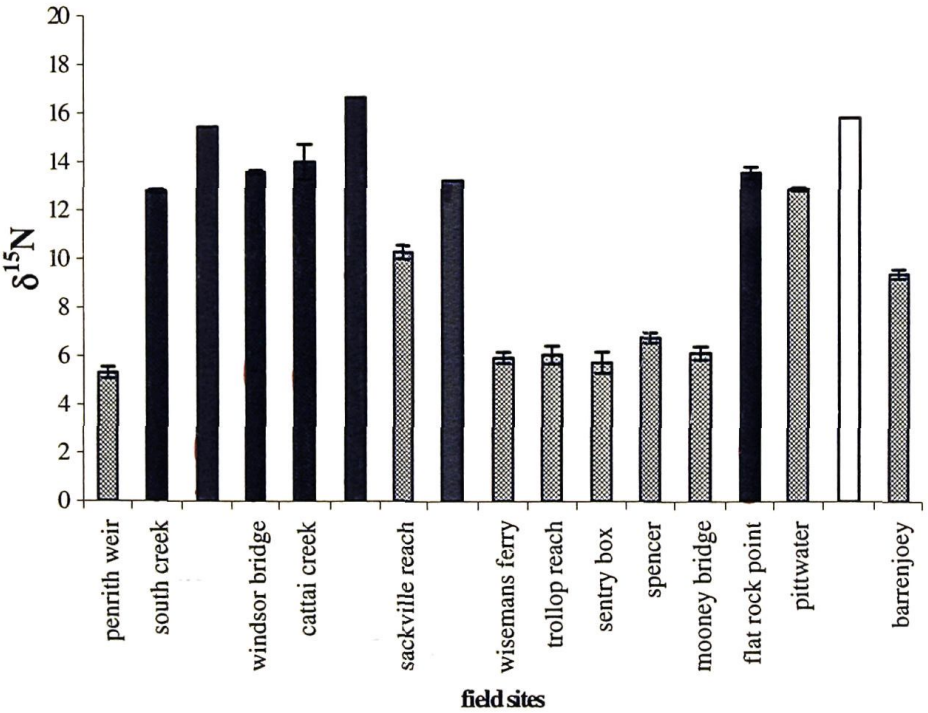

**F.** November 1999



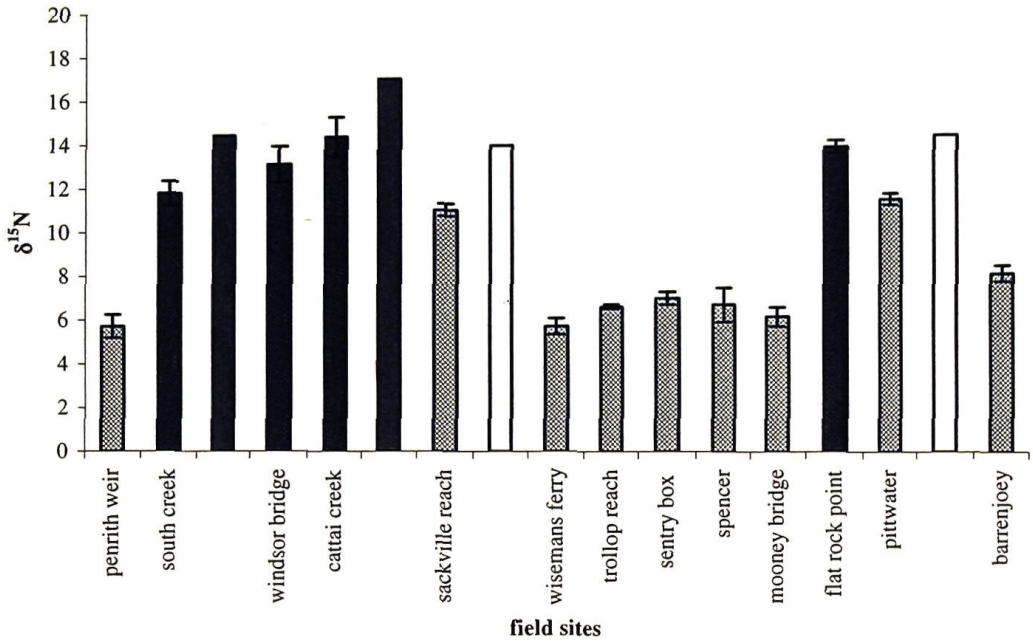
**G.** March 1999




**H.** May 1999



I. June 1999




J. August 1999

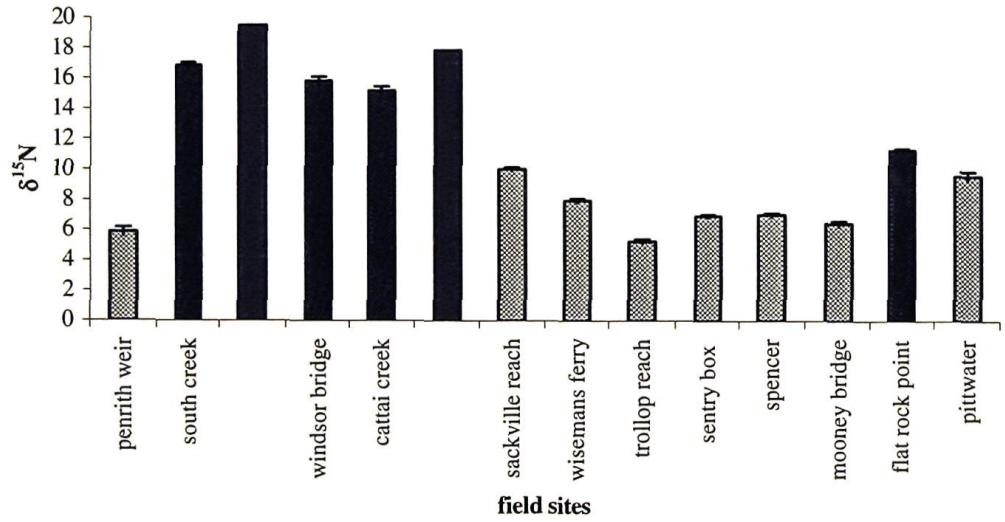



**Figure 5.7:** $\delta^{15}N$ values, as measured by *Microdictyon umbilicatum* bioindicators, at various sites along the length of the Hawkesbury-Nepean River (values are $\delta^{15}N \pm$ SD ‰ for the 6 replicate algae at each location). Values adjusted for the effect of environmental conditions are added (open bars) next to the uncorrected value. Impact sites (STP outfalls) are coloured red.

### *5.4.3 Statistical analysis*

Cochrans test:

C = 0.6161 P< 0.01 This is significant, indicating heterogeneity of variances

Uncorrected results:

| Source | SS | DF | MS | F | P |
|---|---|---|---|---|---|
| TI | 66.156 | 9 | 7.3351 | 1.08 | 0.3852 |
| SI | 8843.2338 | 12 | 736.9362 | 0.00 | 0.0000 |
| AR (SI) | 2.9361 | 13 | 0.2259 | 0.88 | 0.5734 |
| TIxSI | 735.1229 | 108 | 6.8067 | 26.60 | 0.0000 |
| TixAR(SI) | 29.9420 | 117 | 0.2559 | 1.02 | 0.4310 |
| RES | 130.3496 | 520 | 0.2507 | | |
| TOT | 9807.6000 | 779 | | | |

Corrected results:

| Source | SS | DF | MS | F | P |
|---|---|---|---|---|---|
| TI | 66.156 | 9 | 7.3351 | 1.08 | 0.3852 |
| SI | 6938.6442 | 12 | 578.2203 | 0.00 | 0.0000 |
| AR (SI) | 2.9361 | 13 | 0.2259 | 0.88 | 0.5734 |
| TIxSI | 543.7224 | 108 | 5.0345 | 22.13 | 0.0000 |
| TixAR(SI) | 29.9420 | 117 | 0.2559 | 0.96 | 0.4310 |
| RES | 130.3496 | 520 | 0.2507 | | |
| TOT | 7708.8142 | 779 | | | |

SNK tests:

Numbers refer to site numbers, from top of catchment (Penrith Weir, site #1) to estuary (Barrenjoey, Site #13). Sites are presented in this same order in all graphs.

TixSI

1(2)

**1** 1, 5, 9, 13 NS

1(2)

**2** 1 = 2 > 10 >> 5 = 6 = 4 = 8 >> 9 = 7 = 3

1(2)

**3** 5 = 2= 4= 10 = 1 > 7 >> 8 = 3 = 9 >> 6

1(2)

**4** 6 >> rest

1(2)

**5** 10 >> 6 >> rest

1(2)

**6** 7 >> rest

1(2)

**7** all > 8

1(2)

**8** 5 >> rest

1(2)

**9** 9 = 4 = 8 = 3 = 1 =2 = 5 >> 6 = 10 > 7

1(2)

**10** 8 >> 9 = 1 = 3 = 2 = 6 = 7 = 4 = 5 >> 10

| SITE | |
|---|---|
| **1(2)** | |
| **1** | 2 >> 3 >> 4 >> 11 >> 12 = 5 >> 13 >> 8 = 1 = 7 > 6 = 9 = 10 |
| **1(2)** | |
| **2** | 2 >> 3 >> 4 >> 11 >> 12 = 5 >> 13 >>1 = 8 = 9= 7 = 6 = 10 |
| **1(2)** | |
| **3** | 4 >> 11 = 3 >> 2 >> 12 >> 5 >> 13 >> 7 = 8 = 9 = 10 = 6 = 1 |
| **1(2)** | |
| **4** | 4 >> 3 = 11 = 2 >> 12 = 5 >> 13 >10 = 8 = 7 = 9 = 1 = 6 |
| **1(2)** | |
| **5** | 3 >>4 >>2 >> 11 >> 12 = 5 >> 13 >>10 >> 8 > 1 = 7 = 9 = 6 |
| **1(2)** | |
| **6** | 4 >>2 >>11 = 12 = 5 = 3 >> 13 >>8 = 7 = 6 > 9 = 10 = 1 |
| **1(2)** | |
| **7** | 3 >> 4 >> 2 = 12 > 11 > 5 = 13 >> 7 >> 8 > 9 = 6 = 10 = 1 |
| **1(2)** | |
| **8** | 4 = 3 = 11 > 12 = 2 >> 5 >> 13 >> 9 > 10 = 7 = 6 = 8 = 1 |
| **1(2)** | |
| **9** | 4 = 11 >> 3 >> 2 = 12 = 5 >> 13 >> 8 = 9 = 7 = 10 = 1 = 6 |
| **1(2)** | |
| **10** | 2 >> 3 > 4 >> 11 >> 5 = 12 >> 6 = 13 >> 9 = 8 = 10 = 1 > 7 |

**Table 5.4:** Results from an analysis of variance test carried out on $\delta^{15}N$ data collected by *Microdictyon umbilicatum* algae from the Hawkesbury-Nepean River.

| | | *Corrected* | *Uncorrected* |
|---|---|---|---|
| **Factor** | ***df*** | ***MS*** | ***MS*** |
| ***times*** | ***9*** | 7.3351 | 7.3351 |
| ***sites*** | 12 | 578.2204 | 736.9362 |
| ***areas (sites)*** | 13 | 0.2259 | 0.2259 |
| ***SxT*** | 111 | 5.0345 | 6.8067 |
| ***TxA*** | 121 | 0.2559 | 0.2559 |

ANOVA: SxT is significant at $P<0.01$, TxA is not significant for both corrected and uncorrected results(see Appendix 2).

### *5.4.4 The influence of rainfall*

Rainfall had little influence on the $\delta^{15}N$ values of the *M. umbilicatum* bioindicator. After rainfall events, the proportion of N supplied to the river by run-off would be expected to greatly increase (Section 4.1.4). However, the N input from the STPs remained the dominant source at those sites close to outfalls even immediately after rainfall. Significant rainfall events occurred immediately before the August 1997, May 1998, March 1999 and June 1999 sampling events (Figures 5.7a, c, g and i respectively).

The $\delta^{15}N$ values recorded by *M. umbilicatum* immediately after rainfall show little difference in pattern from those at other times, with $\delta^{15}N$ values near sewage outfalls remaining very high. The $\delta^{15}N$ values at sites further away from STPs may have been more affected by rainfall, but these sites varied in ranking in terms of their $\delta^{15}N$ values throughout the entire experimental period (see the SNK tests; Appendix 1). At sites near STP outfalls, $\delta^{15}N$ values remained consistently high at all times, despite the influence of rainfall (Figures 5.7a, c, g and i).

## 5.5 Discussion

For each of the replicate experiments looking at the $\delta^{15}N$ of *M. umbilicatum* bioindicator algae in the Hawkesbury-Nepean River, plants near STPs had very high $\delta^{15}N$ values (Figure 5.7). This indicates that the bulk of their N comes from STP effluent, as the $\delta^{15}N$ values of the algae were approximately the same as that effluent (Figure 5.8). Even as far away as 30km downstream from the nearest STP (e.g. at the Wisemans Ferry site), $\delta^{15}N$ is still elevated above that expected if the bulk of the N in the river came from agricultural run-off alone.

Results from the ANOVA support this hypothesis. Sites were the only factor to show significant variance through time. Areas did not show significant variance (Table 5.2). This is as expected, with the main influence of $\delta^{15}N$ values collected by *M. umbilicatum* being due to the site at which the algae were deployed. SNK tests confirmed the influence of STP effluent at many of the sites. Sites closest to STP outfalls showed no difference in their relative rankings, in terms of $\delta^{15}N$ values, over time. This indicates that the STP effluent is the greatest contributor to the $\delta^{15}N$ at these sites at all times. Other sites showed changes in their rankings, in terms of $\delta^{15}N$ values, over time. Rankings did not show any consistent patterns however, but probably reflect the changing contribution of agricultural run-off, sediment and STP effluent, as well as internal N processes at these sites with time. These changes are masked at sites near STPs by the strong, consistent STP $\delta^{15}N$ signal.

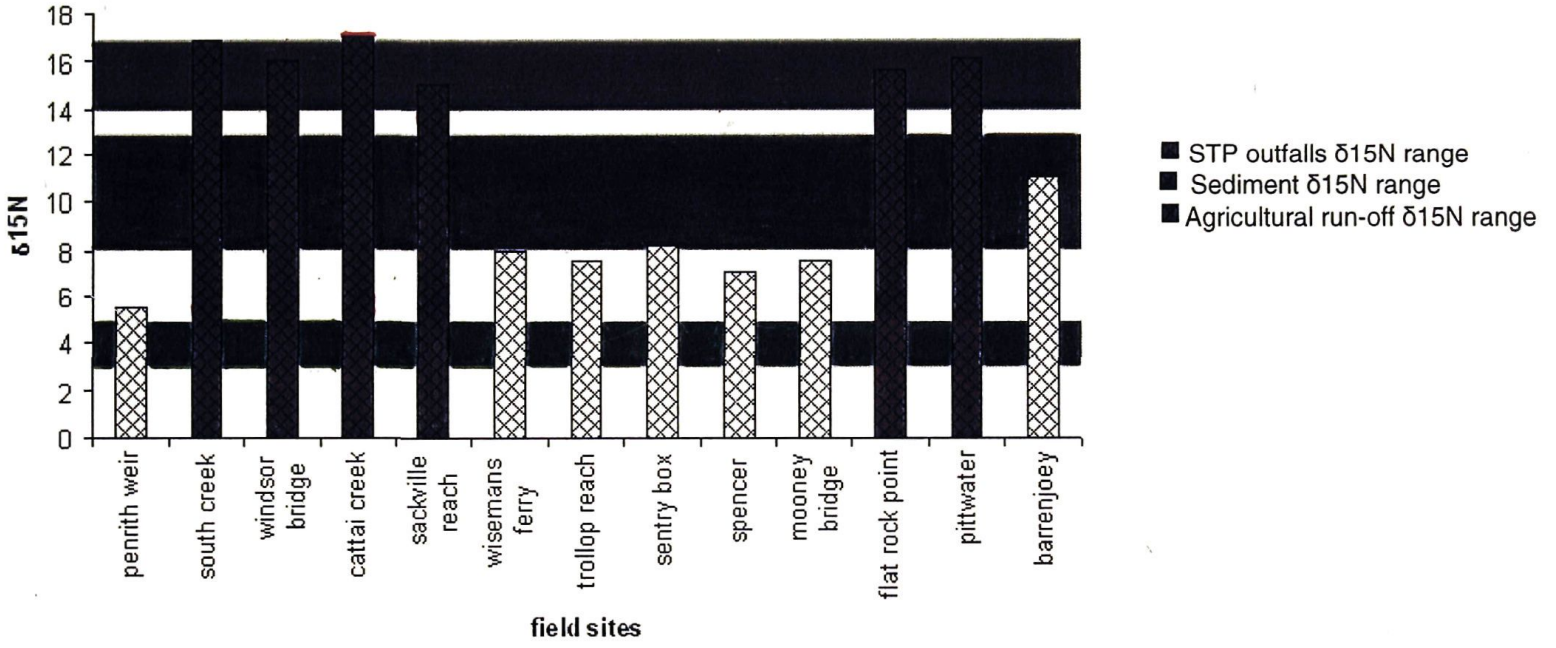



**Figure 5.8**: Mean $\delta^{15}N$ values of *Microdictyon umbilicatum* at each field site, compared to standard $\delta^{15}N$ ranges for STP effluent (red band), agricultural run-off (green band) and sediment (brown band) nitrogen. Sites close to sewage input are coloured red.

Values for the experiments performed using the *M. umbilicatum* bioindicator were found to be very precise (i.e. there was little variance around the mean), much more so than the values from the pilot study (Figure 5.5). The accuracy of the values with respect to the "real" mean cannot be ascertained (see Section 5.1.1). However, estimating possible fractionations in $\delta^{15}N$ between source and sink, and the use of a bioindicator to eliminate other sources of error in $\delta^{15}N$ interpretations would be expected to improve the accuracy of the values collected in the field tests outlined in this Chapter.

Given the precision of the $\delta^{15}N$ values collected with the *M. umbilicatum* bioindicator algae, and the direction of any fractionations identified (all of them negative, and so tending to make final $\delta^{15}N$ values lower, rather than higher), the very high $\delta^{15}N$ values consistently found within 10-20 km of STP outfalls indicate that STP effluent is a major contributor to the overall N load of the Hawkesbury-Nepean River. Variability between replicates (measured by standard deviation) was greatly decreased by the use of the *M. umbilicatum* bioindicator to collect $\delta^{15}N$ data from the Hawkesbury-Nepean River. This can be clearly seen in a comparison in the standard deviations of the results from the pilot study and the main study performed using *M. umbilicatum* (Figures 4.5 and 4.7 respectively). Variation between replicate samples taken in the pilot study may be increased by a large number of factors, including environmental conditions, genetic variability or N stores (discussed in detail in Chapter 2). These sources of error are eliminated or estimated by the use of *M. umbilictum*, and this is reflected in the smaller standard deviations seen in these values.

Rainfall had little effect on the $\delta^{15}N$ of *M. umbilicatum* algae near STP outfalls, again indicating that the contribution of STP effluent to the N load of the river is both large and consistent. Even samples taken immediately after a rainfall event (August 1997, May 1998, March 1999 and June 1999) had very high $\delta^{15}N$ values, indicating that the majority of the N compounds incorporated into the algae was from STP effluent and not agricultural run-off. It

would be expected that the agricultural run-off component would greatly increase after rainfall, but even at these times, STP effluent was still the dominant source of N to the river. Although no attempt was made to characterise the chemical composition of either STP effluent or run-off in detail, these results suggest that agricultural run-off may be largely refractory and therefore less bioavailable than the STP effluent that contains mainly inorganic forms of N ($NO_x$) in the case of STPs in the Hawkesbury-Nepean).

Taking into account the enhanced fractionation in *M. umbilicatum* under low light or high nitrate concentrations increased the final $\delta^{15}N$ estimates at a number of sites, most notably South Creek, Cattai Creek and Pittwater (Figure 5.7). In this study adjusting $\delta^{15}N$ values to allow for differing environmental conditions had little effect on the overall pattern in $\delta^{15}N$ values. This is mainly due to the very high $\delta^{15}N$ values already estimated by the bioindicators at sites close to STP outfalls. Increasing these values further had little effect on the general pattern of high $\delta^{15}N$ near STP outfalls, decreasing at sites further away from outfalls (Figure 5.7). In other studies with less distinct N sources however, allowing for the effect of environmental conditions on N fractionation in a bioindicator species may be crucial to determining the main source of N to the system. This is discussed further in Chapter 6.

Using *M. umbilicatum* as a "bioindicator" to collect $\delta^{15}N$ data from the Hawkesbury-Nepean, and estimating fractionation under various conditions has greatly reduced experimental error in this study, allowing source $\delta^{15}N$ and identity to be clearly established. The $\delta^{15}N$ data collected using the *M. umbilicatum* bioindicator algae for a period of 3 years indicates that, over this time period, the main source of N contributing to the eutrophication of the Hawkesbury-Nepean River was effluent from sewage treatment plants.

# CHAPTER 6: GENERAL DISCUSSION.

## 6.1 Major findings

The research reported in this thesis was designed to investigate the flux of nitrogen from different catchment and in-stream sources to primary producers in a coastal river and estuary, the Hawkesbury-Nepean River, NSW, Australia. The nitrogen stable isotope $^{15}N$, measured as $\delta^{15}N$, was used as a tracer of bioavailable N sources. In order to evaluate the contribution of the different sources investigated, sewage treatment plants, agricultural runoff and recycled N from sediments, an algal bioindicator, *Microdictyon umbilicatum*, able to be deployed in the waterway, was developed. In addition, the bioindicator was used in a number of laboratory experiments to test the effect of various environmental factors on $^{15}N$ incorporation into biomass. This allowed the field data to be calibrated, eliminating potential errors in the interpretation of $\delta^{15}N$ data commonly found in "tracer" studies.

The influence of environmental conditions on the level of fractionation in *M. umbilicatum* was found to be large, with light level and ambient nitrate concentration increasing fractionation markedly from the normal discrimination by this species. Characterisation of the main sources of N in terms of their $\delta^{15}N$ values was shown to be crucial to the use of $\delta^{15}N$ data as a tracer of N source. In the Hawkesbury-Nepean River, each of the main N sources was found to be distinct, allowing $\delta^{15}N$ data to be interpreted in terms of the main N source contributing the to $\delta^{15}N$ of the bioindicators at each site in the river.

Effluent from STPs was found to be the major source of bioavailable N to the Hawkesbury-Nepean River, and was the main source of N to the *M. umbilicatum* algae at all sites near STPs and up to 30km downstream. Agricultural run-off was an important source of N at sites further away from STP outfalls, but was not a constant influence, due to the importance of rainfall events to this source.

The development of the *M. umbilicatum* algal bioindicator provides a technique to eliminate many of the problems associated with $\delta^{15}N$ "tracer" studies. In addition to mapping critical pieces of the nitrogen cycle of an estuary that is influenced heavily by human activities, this thesis developed a technique that allows interpretation of $\delta^{15}N$ data as a tracer of N source in aquatic ecosystems with increased increase precision and greatly reduced error. This method could be easily adapted to answer important questions about N source in a wide range of aquatic ecosystems.

## 6.2 Limitations to the interpretation of $\delta^{15}N$ results and their solutions

For the vast majority of $\delta^{15}N$ tracer studies, there are a large number of potential errors, which may confound the interpretation of $\delta^{15}N$ values. Many complex reactions are involved between the source and sink of N, all of which have the potential to affect stable isotope ratios.

To correctly identify sources of N using $\delta^{15}N$ data, a number of criteria must be met:

1. The $\delta^{15}N$ values of the source(s) must be known
2. Fractionation(s) occurring between source and sink (the organism being analysed) must be quantified
3. Any fractionation or partitioning of N occurring in the organism itself must be quantified
4. The effects of environmental conditions on these processes must be understood
5. Errors due to selection, processing or analysis of the samples must be allowed for.

Stable isotope ratios are affected by a large number of factors, many of which are not controlled or estimated in traditional tracer studies. In this thesis, the green alga *Microdictyon umbilicatum* was used as a bioindicator alga to collect $\delta^{15}N$ data from the Hawkesbury-Nepean River. Starting with a genetically and physiologically homogeneous "receptor" organism eliminates many of the problems associated with collecting biological material, even of the same species, from the field. Use of the cloned *M. umbilicatum* algae removed many of the confounding factors associated with trophic level, species differences, genetic variation between individuals or sample selection. However, the use of a bioindicator did not remove the effect of environmental conditions and N turnover, so the effect of these parameters was estimated through a series of experiments and was allowed for in interpreting the field results when necessary.

The use of *M. umbilicatum* as a bioindicator allows the effect of environmental conditions on N turnover to be investigated and quantified, and provides a mechanism to link laboratory and field data. This provides a technique to eliminate many of the criticisms leveled by investigators at $\delta^{15}N$ "tracer" studies (e.g. Mariotti *et al.*, 1981; Bustamante and Branch, 1996; Handley and Scrimgeour, 1997). This thesis provides a technique, in the form of the *M. umbilicatum* bioindicator, to increase precision and greatly reduce error in the interpretation of $\delta^{15}N$ data as a tracer of N source in aquatic ecosystems. However, use of the bioindicator also needs to be linked to data that characterise the $\delta^{15}N$ of the main sources of N to an ecosystem, and environmental factors affecting fractionation in order to provide meaningful data.

### *6.2.1 Environmental factors affecting fractionation*

In tracer studies, it is important to estimate any discrimination between N source and sink. The size and direction of any fractionation can be heavily influenced by environmental conditions. Four environmental parameters were found likely to affect the fractionation of N in *M. umbilicatum* (Chapter 3):

1. The amount of discrimination that takes place in plants and algae is, in general, positively correlated with the concentration of $NO_3^-$ in the growth medium. This was also found to be the case in *M. umbilicatum*.
2. The rate of N uptake, and hence discrimination, is inherently linked to illumination levels. In *M. umbilicatum*, low ambient light levels resulted in a marked increase in discrimination.
3. Plant and alga growth rates, enzymes and the uptake of $NH_4^+$ and $NO_3^-$ are all affected by temperature. However, in the temperature ranges encountered by the *M. umbilicatum* algae in the Hawkesbury-Nepean, temperature had no effect on the level of discrimination.

4. Salinity may influence the relative uptake of $NH_4^+$ and $NO_3^-$ in plants and algae, and hence affect their $\delta^{15}N$ but *M. umbilicatum* showed no change in discrimination due to salinity, possibly because of the algas ability to efficiently osmoregulate.

In order to reduce error due to changes in discrimination resulting from environmental factors, parameters found to affect discrimination (nitrate concentration and light levels) were monitored while *M. umbilicatum* algae were in the field and $\delta^{15}N$ values adjusted for their influence if necessary. The mechanisms responsible for discrimination under low light or high nitrate conditions are discussed in Sections 2.4.4 and 2.4.3 respectively.

Other factors found by other researchers to affect $\delta^{15}N$ values in plants, such as time of day and season, nitrogen fixation, uptake of gaseous ammonia, water availability and associations with mycorrhizal fungi are discussed further in Section 2.4.7. These factors were considered unlikely to affect fractionation in *M. umbilicatum* and hence were not investigated further in this study.

Corrections for enhanced fractionation in *M. umbilicatum* under low light or high nitrate concentrations increased the final $\delta^{15}N$ estimates at a number of sites (see Figure 5.7). If both low light and high nutrient conditions were encountered by *M. umbilcatum* at a site, these effects were considered to be additive, and the combined fractionation of +5.62‰ (Section 3.5) was added to the final $\delta^{15}N$ value of the algae from the relevant site. This was at best a crude estimate of the combined effects of these two parameters, and would require further investigation if the bioindicator was to be used as a tool in ecosystems with less clearly defined possible N sources and field results than the Hawkesbury-Nepean.

In this study, adjusting $\delta^{15}N$ values to allow for differing environmental conditions had little effect on the overall pattern in $\delta^{15}N$ values, mainly due to the very high $\delta^{15}N$ values already estimated by the bioindicator algae deployed at sites close to STP outfalls. Increasing these values further had little effect on the general pattern of high $\delta^{15}N$ near STP outfalls, decreasing at sites further away from outfalls. However, in other studies with less distinct N sources, allowing for the effect of environmental conditions on N fractionation in a bioindicator species may be crucial in reducing error to a level where the main source of bioavailable N to an ecosystem can be confidently determined.

### *6.2.2 Nitrogen turnover in M. umbilicatum*

The rate of exchange between an organism and its environment can also significantly affect its final $\delta^{15}N$ signal. Stored N compounds reflect the nitrogen environment of the organism at the time the compound was formed, and dilute any N uptake from the current environment. The final $\delta^{15}N$ of a plant or alga therefore reflects the balance of the $\delta^{15}N$ of the remaining and new nitrogen compounds, giving a distorted reflection of the $\delta^{15}N$ of the environment. An internal control to allow for any bias due to stored N compounds was therefore included in the *M. umbilicatum* bioindicator, by growing algae in a known N source in the laboratory (see Section 3.4.1 for more detail).

### *6.2.3 Charactering the $\delta^{15}N$ of the main nitrogen sources*

In order to use $\delta^{15}N$ values as a "tracer", it is essential to characterise the $\delta^{15}N$ values of any possible N sources. For the Hawkesbury-Nepean River, STP effluent, agricultural run-off and N recycled from the sediments were characterised in terms of their $\delta^{15}N$. Each source was found to have a distinct $\delta^{15}N$, which could be used in the interpretation of $\delta^{15}N$ data from the *M. umbilicatum* bioindicators in the field experiments. Although there are some crude approximations used to deal with complex factors such as additive effects and sediment recycling of nitrogen, and other factors are ignored (for example, the effect of modified flow due to the experimental enclosures on diffusion rates into the algae), it is felt that these are not a barrier to the use of this technique to determine the main sources of bioavailable N to the Hawkesbury-Nepean system.

*STP effluent*

Mean $\delta^{15}N$ values in M. umbilicatum algae grown near STP outfalls and affected by STP effluent and those grown in river reaches mainly influenced by agricultural run-off were highly characteristic (distinct with low variance), and varied little over the 3 sampling periods. These values accurately reflect the $\delta^{15}N$ of these sources, as they were measured in the field at the point where each source entered the river. Bioavailable N contained in effluent was found to have a very high $\delta^{15}N$, (consistently greater than 14.02‰) as would be expected to result from the large number of processes in the treatment plant, all of which discriminate against the lighter isotope and enrich effluent in $^{15}N$ (section 4.4.1).

The aim of this experiment was not to distinguish between different STPs, but to identify a mean "characteristic STP $\delta^{15}N$" for use in identifying the main sources of N contributing to the eutrophication of the Hawkesbury-Nepean River. A "characteristic" $\delta^{15}N$ of STP effluent was able to be determined for this river system, due to both the relatively low range of values between different STPs and the distinctive high $\delta^{15}N$ of all these samples. This is clearly related to the fact that all STPs in the system have similar rates of sewage treatment. This may not be the case in other aquatic systems and the complex issue of determining "characteristic" $\delta^{15}N$ values for STP inputs would have to be approached on a case-by-case basis.

*Agricultural run-off*

*M. umbilicatum* grown in run-off from agricultural land into the Hawkesbury-Nepean River had a relatively low $\delta^{15}N$, consistently < 5.18‰. This $\delta^{15}N$ is a product of a blend of nitrogen sources originating from animal wastes and inorganic fertiliser from the intensive horticulture and animal production in areas in the catchment, and any subsequent fractionations undergone by these compounds before entering the river (Section 4.4.2).

There was some difference between sites, probably due to some differences in the type of activity, and hence composition of the run-off, between these sites. However, this difference between sites was within the limits of variance seen within each site, and was therefore of little significance. Variance over time was also greater than variance between sites, indicating that differences in the composition of run-off between sites were not particularly important in the Hawkesbury-Nepean and did not preclude determining a "characteristic" $\delta^{15}N$ for run-off.

This may not be the case in other aquatic ecosystems where it is desirable to measure the contribution of bioavailable N from run-off, and each situation would have to be assessed individually.

*Sediment*

The $\delta^{15}N$ data characterising N from the sediments in the laboratory was much more variable than data from the other sources, with outlying data points overlapping the values characteristic of STP effluent and agricultural run-off. Sediment processes involving N are extremely complex, and are influenced by a number of factors including oxygen and carbon inputs (Section 4.4.3). However, it was not the aim of this thesis to investigate the N processes occurring in the sediments, but rather to simply gain a "characteristic" $\delta^{15}N$ for N recycled from the sediment in the Hawkesbury-Nepean River, and determine if this was the source of the high $\delta^{15}N$ values consistently measured at many sites along the river during the course of the project.

Although the methods use to determine the $\delta^{15}N$ values of algae grown in water enriched with sediment pore-water N were relatively crude, the values measured in this study fell within the range of values measured in sediments using more refined methods, such as chambers (Berelson *et al.*, 1996; Heggie *et al.*, 1998). Despite the limitations of determining a characteristic $\delta^{15}N$ for sediment, it was felt that the main sources of bioavailable N to the Hawkesbury-Nepean River could still be confidently determined, due to the very distinctive and constant pattern of $\delta^{15}N$ values found in the field study (Section 5.4.2).

## 6.3 Interpretation of $\delta^{15}N$ values in terms of the main sources of bioavailable nitrogen to the Hawkesbury-Nepean River.

The $\delta^{15}N$ data collected using the *M. umbilicatum* bioindicator algae for a period of 3 years indicates that, over this time period, the main source of bioavailable N entering the Hawkesbury-Nepean River was effluent from sewage treatment plants.

The $\delta^{15}N$ values collected with the *M. umbilicatum* bioindicator algae were very precise, and error is extremely unlikely to account for the high $\delta^{15}N$ values over this period of time. The direction of all fractionations between N sources and *M. umbilicatum* identified was negative, tending to make final $\delta^{15}N$ values lower, rather than higher. Using *M. umbilicatum* as a "bioindicator" to collect $\delta^{15}N$ data from the Hawkesbury-Nepean, and estimating fractionation under various conditions has greatly reduced experimental error in this study, allowing source $\delta^{15}N$ and identity to be clearly established. The very high $\delta^{15}N$ values consistently found near STP outfalls are therefore extremely likely to be due to the $\delta^{15}N$ of the effluent, and not to other extraneous factors.

If there are 3 or more sources, it is almost impossible to accurately determine the exact proportion of each N source to the overall $\delta^{15}N$ of an organism, even if the $\delta^{15}N$ of each source is distinct (for review, see Shearer and Kohl, 1993). However, if the $\delta^{15}N$ of an organism is very close to that of one source, it can be assumed that this is the main N source used by the organism. Of course, this can be confounded by fractionation between the source and the organism. However, as fractionation in *M.umbilicatum* under various conditions was measured and corrected for in field experiments, this is unlikely in this study, indicating that the main source of bioavailable N in the Hawkesbury-Nepean was STP effluent.

Modelling may be used to estimate the maximum potential input from each source to the overall N at any site with some success, as has been done by Phillips and Gregg in US systems (Phillips and Gregg, 2001; Phillips and Gregg 2003). However, these models still do not give definitive answers, only a small number of plausible solutions. It is beyond the scope of this thesis to attempt this type of complex modelling.

N may occur in many different chemical forms in aquatic environments, but it is the dissolved inorganic forms that are the most readily available for assimilation by algae i.e. are bioavailable. It is these forms of N that largely fuel the problematic excess plant growth and algal blooms in eutrophic systems. Hence, even though there may be a larger amount of total N entering the system from other sources, it is the source of bioavailable N which is important in management terms.

*The influence of rainfall*

Rainfall had little effect on the $\delta^{15}N$ of *M. umbilicatum* algae near STP outfalls, indicating that the contribution of STP effluent to the bioavailable N load of the river is both large and consistent. Even samples taken immediately after a rainfall had very high $\delta^{15}N$ values, indicating that the majority of the N compounds incorporated into the algae was still from STP effluent and not agricultural run-off. Despite diffuse sources of nutrients usually dominating the input loads to rivers and estuaries, both in Australian and northern hemisphere systems, it is difficult to interpret the ecological importance of these sources simply by their size. For example, Eyre *et al.* (1997) found that the Richmond River, in northern New South Wales, Australia, had no change to N concentrations in water and sediments and low chlorophyll *a*, despite nitrogen loads (all from diffuse sources) increasing by a factor of 3 over the past 50 years. In contrast, the nearby Brunswick River receives about 10% of its nutrient load from STP effluent and has frequent phytoplankton blooms. The majority of run-off N

enters the Hawkesbury-Nepean after heavy rainfall events, and these episodic inputs, although high in total N load, are often composed of refractory (non-bioavailable) forms of N which travel rapidly through the river system, ending up in an estuarine sink with little contribution to eutrophication effects in the river itself. In contrast to this pulsed, refractory N source, the chronic, bioavailable N delivered by STP effluent was the dominant source of N to the algae in the river.

*Sediments*

Bottom sediments in shallow-water aquatic ecosystems may act as both a nutrient sink and a source of regenerated nutrients. Initial pilot studies in the Hawkesbury-Nepean river indicate that denitrification is not a major source of N loss, with denitrification removing less than 1% of the total $NO_x$ load downstream of a sewage treatment plant (Hanington, unpublished).

However, studies by Heggie *et al.* (1999) in Port Phillip Bay found denitrification levels in this estuary were high, as long as sediment respiration rates were low. Other factors, such as drying, can also affect sediment processes. Sediment N cycles are complex and still poorly understood in Australian ecosystems, and the contribution to or removal of bioavailable N loads by sediments is a largely unknown quantity.

Nitrogen compounds recycled from the sediments may confound the $\delta^{15}N$ results at many of the sites in the study, as their $\delta^{15}N$ was quite variable, and in a mid-range between STP and agricultural run-off $\delta^{15}N$. The relative contribution of sediment N to sites with $\delta^{15}N$ values in the range of 6-12‰ could not be determined. The input of N compounds from the sediment could not be distinguished from the effect of varying proportions of agricultural run-off and STP effluent either. However, it was not felt that this was a major obstacle to the

interpretation of the $\delta^{15}N$ results in the present study as there were so few of these sites (see Section ).

Despite limitations to the interpretation of $\delta^{15}N$ values in this study due to multiple N sources, STP effluent is identified as a large and consistent contributor to the overall N available to plants and algae in the Hawkesbury-Nepean River, and the main source of bioavailable N at sites near STP outfalls.

This is consistent with findings in some other Australian river systems. For example, in dry years, STP's and other point sources are significant inputs of both nitrogen and phosphorus in the Murrimbidgee River, but diffuse sources are dominant in other years (Davis & Koop, in press).

## 6.4 Implications of the findings of this study to the management of the Hawkesbury-Nepean and similar systems.

### *6.4.1 Other factors and their influences on eutrophication in Australian rivers*

It is not only the quantity of nutrient entering rivers that is important, but also the timing, location and nature of the nutrients. Rainfall variability on the Australian continent is high, and linked to variation in the *El Niño / La Nina* cycle and Southern Ocillation (ENSO) events (Harris, 1996, Davis & Koop, in press). This leads to periods of extremely high flow in rivers (*La Nina*) being interspersed with periods of very low, or even zero, flow (*El Niño*). This greatly affects the nutrient cycles as well as the overall ecology of the river. In periods of low flow, nutrients delivered to the river will often lead to the development of algal blooms. The same amount of nutrient entering a fast-flowing river system may have no effect on algal

growth as it is quickly removed to the estuary and inshore coastal waters. Here, processes of dilution, dispersion, sedimentation and denitrification (assuming conditions still exist for this process to take place; see Section 4.4.3) reduce the amount of bioavailable N. The residence time of nutrients, and the difference between the effective (i.e. the fraction of the nutrient load that actually enters the nutrient cycle and contributes to eutrophication) and absolute (i.e. the total nutrient load entering the system) nutrient loads is extremely important when assessing the relative impacts of nutrient sources (Davis and Koop, 2001).

Diffuse sources of nutrients usually dominate input loads to rivers and estuaries, both in Australian and overseas. For example, in the Chesapeake Bay system in the USA, diffuse sources were found to contribute approximately 60% of the total N to the Bay, whereas point sources contributed only 28% of the TN load (Boynton *et al.,* 1995). In the Hawkesbury-Nepean, previous studies have estimated that diffuse sources contribute 70-80% of the TN, with point sources contributing only 20-30% (Davis *et al.,* 1998). However, the ecological importance of point and diffuse sources is not simply related to their size. Point sources such as STPs deliver a constant discharge to a particular location, while most diffuse sources are heavily related to episodic storm events (Eyre *et al.,* 1997; Davis and Koop, in press). For example, in a period of low flow in 1994, it was calculated that 84% of the nitrogen delivered to the Hawkesbury –Nepean River at this time was from STPs (Kerr, 1994). A large algal bloom also occurred in the river at this time. In other studies on temperate Australian rivers, the proportion of diffuse nutrients immediately exported to the ocean in wet weather has been estimated at approximately 30% of the total diffuse load (SKM, 1997). On the other hand, rivers with heavily reduced flows due to dams, irrigation or drought conditions may have very long residence times, affecting sediment-water interactions, water stratification and turbidity and ecological processes occurring in the river.

A study comparing two NSW rivers by Eyre *et al.* (1997) found that the Richmond River, which receives only diffuse N sources, is not subject to algal blooms despite N loads increasing by a factor of 2.5 over the last 50 years. In contrast, the Brunswick River, which receives approximately 10% of its TN from point sources (STPs), was subject to frequent algal blooms. This raises the question of the bioavailability of nitrogen from the two sources. N entering the system from STP outfalls may be in a form much more readily available to aquatic plants, hence contributing disproportionately to their growth.

Other factors, such as light, also influence plant and alga growth. Blackwater streams and waterways with high turbidity restricting light penetration may have no algal blooms, even though other factors precipitating blooms, such as high nutrient loads, may be present.

Stratification of the water column in periods of low flow is common in Australian river systems. Wind also controls stratification in some water bodies, with wind movement of surface waters preventing stratification. Once the water column is stratified, cyanobacteria quickly gain a competitive advantage due to their ability to regulate buoyancy, unless other factors, such as zooplankton predation, nutrient availability or light levels limit these blooms. If stratification persists for long enough, anoxic conditions may develop in the sediment layer, prompting release of nutrients to the water column.

Zooplankton grazing can be an important control on algal populations in some ecosystems, but attempts to manipulate food webs in order to reduce blooms of nuisance phytoplankton have largely met with long-term failure, with some studies (e.g. McQueen, 1998) finding that initial results are not sustained and eventual algal biomass is as high or higher than before biomanipulation began.

### *6.4.2 Management recommendations for the Hawkesbury-Nepean river system*

Effluent from the many sewage treatment plants discharging into the Hawkesbury-Nepean river was found to be a large and consistent source of bioavailable N to the system. A vigorous scientific debate about the relative importance of point and diffuse sources to eutrophication, as well as differences about which of the nutrients, nitrogen, phosphorus, or both need to be managed has made it difficult for managers to decide on nutrient reduction strategies, particularly given the high cost of implementing these decisions.

Increasingly, the importance of residence times (i.e. flow regimes) on river systems is being recognised as a key parameter in eutrophication in Australia (Davis and Koop, in press). Flow manipulation, both to reduce nutrient residence times and prevent stratification (and associated anoxia of the sediments), may be an effective management tool in the Hawkesbury-Nepean, but is made more difficult by the demands on security of water supply to the city of Sydney with its approximately 4.5 million inhabitants. The Warigamba dam, the city of Sydney's main source of water, is situated in the upper reaches of the river and decisions about using flow manipulation to reduce risks of eutrophication need to be offset against security of drinking water supply.

The contribution of sediments to bioavailable N in the Hawkesbury-Nepean is presently largely unknown, and this study has not fully resolved this question. Management solutions to this complex issue are still being developed and largely rely on trying to maintain an oxygenated layer over sediments to prevent nutrient release, dredging or dosing with alum or iron salts.

The results of this study make clear that in the Hawkesbury-Nepean, it is STP effluent that provides the bulk of bioavailable nitrogen. Above all, reducing the nutrient load from this source, coupled with other techniques such as flow regulation and catchment management to prevent run-off from agricultural lands, particularly during episodic rainfall events, would be an effective way of reducing the effects of eutrophication on the river system.

### 6.5 Wider use of the technique developed in this thesis.

The most important contribution of this thesis is in its development of a useful method of using $\delta^{15}N$ data to determine the main sources of N to an aquatic ecosystem in Australia. This has concentrated on two main problems often encountered in such studies: the elimination of differences in response variables (such as genetic variability and physiological responses) in test organisms collected from the field by developing a bioindicator alga that can be easily grown in the laboratory and deployed on a wide spatial scale in the river/estuarine system; and quantification of environmental factors on the bioindicator alga so that field data can be calibrated for standard environmental conditions. The use of $\delta^{15}N$ data as a tracer of nutrient source has been heavily criticized in the past, with many researchers pointing out that the size of confounding factors makes it impossible in most cases to determine N sources using $\delta^{15}N$ values. However, stable isotopes allow the distinction of identical N compounds from different sources, and so have many uses in studies of this type if extraneous errors can be controlled.

One of the main criticisms of $\delta^{15}N$ tracer studies has centred around the issue of confounding factors and cases such as the Hawkesbury-Nepean River with large, distinct N sources are thought to be the exception rather than the rule (Van Dover *et al.*, 1992; Shearer and Kohl, 1993, Handley and Scrimgeour, 1997). In other studies with less distinct N sources, variation caused by environmental changes may be large enough to mask small differences in source $\delta^{15}N$. In these ecosystems, allowing for the effect of environmental conditions on N fractionation in a bioindicator species may be crucial to determining the main source of bioavailable N to the system.

This thesis has rigorously developed and explored the use of a bioindicator species to collect $\delta^{15}N$ data from aquatic ecosystems, and has eliminated or quantified many of the sources of error commonly confounding the interpretation of $\delta^{15}N$ data. It has contributed key improvements to the use of $\delta^{15}N$ values as a tracer of the main sources of bioavailable N to an ecosystem, and greatly increased the accuracy and precision of this technique. The methods developed in this thesis are suitable for assessing the sources of N pollution in rivers, estuarine and marine waters in Australia.

This thesis also provides a theoretical framework for the development of $\delta^{15}N$ bioindicators suitable for tracing nutrient sources in other ecosystems. If the appropriate ancillary data are collected and fractionations between source and sink are accounted for, $\delta^{15}N$ values collected by a well-characterised bioindicator, as outlined in this study, can provide a powerful tool for examining the sources and sinks of nitrogen compounds in a variety of ecosystems.

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