Quantitative ecological impact assessments using natural abundance carbon and nitrogen stable isotope signatures

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Abstract

The use of $\delta^{15}N$ and $\delta^{13}C$ signatures to infer sources of enrichment in ecological systems relies on predictability in the transfer of $\delta^{15}N$ and $\delta^{13}C$ ratios. This thesis examines patterns of $\delta^{15}N$ and $\delta^{13}C$ change as pools of nitrogen and carbon move from a sewage effluent discharge into organisms in an adjacent coastal rocky reef community (Titahi Bay, New Zealand). These changes and their mechanisms are examined in the broader context of current research using carbon and nitrogen stable isotope ratios in marine ecology, with particular reference to impact assessment.

Firstly this thesis examines the assimilation of nitrogen and carbon isotopes in *Ulva* sp. under varying light conditions and nitrogen source (e.g., nitrate or ammonium). In a field study, algae grown at depth and under lower light conditions showed comparatively lighter nitrogen isotope signatures relative to the predicted concentration of available ^{15}N-enriched sewage nitrogen. In a complementary laboratory experiment, results from manipulated light availability and N source (either nitrate or ammonium, in equivalent molar concentrations) suggest that: 1) low-light conditions can produce algae with lighter nitrogen isotope signatures; and 2) this effect was more pronounced for ammonium (3.7 ‰ difference between high light and low light treatments) than for nitrate (0.6‰ difference between high light and low light treatments) sources. Stable carbon isotope ratios ($\delta^{13}C$) of *Ulva* sp. grown in conditions of low nitrogen availability were shown to be generally lower than those grown in nitrogen rich conditions in both field and laboratory studies. Where nitrogen supply was sufficient for growth, low light conditions also produced generally lower $\delta^{13}C$ signatures than high light conditions. Experimental trials with a uniform

dissolved inorganic carbon source and altered light and nitrogen enrichment levels produced $\delta^{13}C$ levels in *Ulva* sp. tissue that spanned the recorded $\delta^{13}C$ ranges of many common algal species; -5.99‰ (high light, with added ammonium and phosphate) to -17.61‰ (high light without nutrient additions). Chapter 3 of this study examines the growth response of *Ulva* sp. to surplus nitrate and ammonium (the two most common forms of nitrogen available to plants in seawater), under light limited conditions. *Ulva sp.* experienced a temporary reduction in growth rate and nitrogen assimilation capacity (shown in tissue nitrogen indices) when grown on nitrate, relative to ammonium. The magnitude and the temporary nature of these results suggest that in natural populations the relative proportion of nitrate or ammonium available is unlikely to significantly affect the growth capacity of *Ulva* sp.. In chapter 4, I use $\delta^{13}C$ and $\delta^{15}N$ signatures to separately trace the dissolved and particulate fractions of sewage effluent dispersal onto a rocky reef community. $\delta^{15}N$ signatures from tissue of the macroalga *Carpophyllum maschalocarpum*, and the herbivorous isopod *Amphoroidea media* tracked the distribution and signature of DIN from a sewage treatment plant that generated heavy $\delta^{15}N$ signatures. $\delta^{13}C$ signatures from tissue of the filter-feeding half-crab *Petrolisthes elongatus* tracked the distribution and signature of suspended sewage particulate organic matter.

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Table of contents

Chapter 1 – General introduction

Overview

Coastal marine environments are comprised of a wide variety of species, and many of these are of conservation and economic interest. The dynamics and structure of these diverse communities are often regulated by complex networks of trophic linkages (Walters and Moriarty 1993, Hart and Lovvorn 2003, Levin et al. 2006). Food webs in coastal marine environments are influenced by nutrients of both marine and terrestrial origin (Cole and Caraco 2001). Terrestrial sources include natural terrestrial organic matter, such as leaf litter delivered via streams and rivers, as well anthropogenic sources such as sewage and agricultural runoff (Kwak and Zedler 1997, McClelland and Valiela 1998b, Chanton and Lewis 2002).

Changes in the levels of nutrient availability and the physical and chemical properties of the marine environment have been shown to cause changes in trophic pathways and abundances of species in an ecosystem (Heip 1995, McClelland and Valiela 1998a, Proulx and Mazumder 1998, Bokn et al. 2002, Tewfik et al. 2005). The adaptation of some marine communities to low levels of some nutrients has made them susceptible to anthropogenic effects.

Increasing human populations in coastal areas worldwide have caused increases in environmental and ecological change. These changes include alteration and destruction of habitats and ecosystems, eutrophication, the decline of fish stocks, and

changes in sediment flows. Coastal ecosystems are also at risk of damage from human-derived pollutants, such as pesticides, hydrochlorides, heavy metals such as lead, and petrochemicals from roads (GESAMP 2001).

One of the principal impacts of human population growth is the increased production of nitrogen and phosphorus. Often, these nutrient subsidies find their way into estuarine and marine food webs. In most marine environments, addition of these nutrients leads to increased primary production (Pedersen 1995, Valiela et al. 1997). This can be detrimental to coastal ecosystems in several ways. Increased nutrient levels can lead to increased biomass of algae with fast nitrogen uptake and assimilation rates, and may lead to displacement of other algal species (Valiela et al. 1997). The decay of excess primary production (e.g., phytoplankton and fast-growing macroalgae) can lead to an increase in the consumption of oxygen in water and in bottom sediments, leading to mortality of benthic marine organisms (Heip 1995, Cloern 2001, Gray et al. 2002). Increased nutrients can also favour the excessive growth of toxic algae, which may cause mortality of higher organisms and contamination of seafood (Keller and Rice 1989, Butler et al. 1995). It is generally accepted that peak seasonal growth of seaweeds in temperate marine environments is most often limited by the availability of nitrogen (Hanisak 1983, Fujita et al. 1989, Peckol et al. 1994).

In coastal marine systems, nitrate (NO_3^-) and ammonium (NH_4^+) are typically the most abundant forms of inorganic N in seawater. The majority of nitrogen in coastal environments worldwide is available to marine macroalgae in the form of nitrate, which is typically present in concentrations between 0 and 30 µM (DeBoer 1981,

Sharp 1983). Ammonium is most often available in concentrations below 3 µM, but can be increased by terrestrial sources of enrichment, such as sewage outflows. Nitrite concentrations typically do not exceed 5% of dissolved inorganic nitrogen (DIN) in seawater (Sharp 1983). Organic sources of nitrogen available in minor quantities to macroalgae in coastal environments are urea (Hanisak 1983), and amino acids (DeBoer 1981).

Ammonia is present in seawater in equilibrium between gaseous ammonia (NH_3) and dissolved ammonium ions (NH_4^+). Algae take up ammonium ions by active transport across the plasma membrane. Gaseous ammonia is taken up through passive diffusion across this membrane. Nitrate ions also require active transport during uptake (Syrett 1981). However, ammonium is typically taken up into macroalgae in higher rates than nitrate (Wallentinus 1984, Thomas & Harrison 1985) and ammonium uptake may inhibit the uptake of nitrate in some species, including *Ulva* sp. (Hanisak, 1983, Thomas & Harrison 1987, Rees et al. 2007).

Nitrate requires reduction to nitrite then ammonium prior to assimilation. These steps are catalysed by nitrate reductase and nitrite reductase, and have energy requirements associated with the production of these enzymes as well as the reduction reaction. Because of this energy requirement, rates of nitrate uptake typically vary under changing light conditions, and this relationship follows a rectangular parabola (Wheeler 1982). Ammonium uptake rates in macroalgae are typically unaffected by irradiance (Wheeler 1982, Duke *et al*. 1989).

Ammonium is assimilated into algal tissue by enzymatic conversion to amino acids via the glutamine synthetase / glutamine : 2-oxoglutarate aminotransferase pathway (Lea & Miflin, 1974). This process is linked to photosynthesis due to the requirement of 2-oxoglutarate for the conversion of glutamine to glutamine to glutamate, as well as a requirement for ATP (Galvez et al. 1999).

Both nitrate and ammonium may be stored in algal tissue prior to assimilation. Nitrate has been observed to be stored in unreduced intracellular reserves during winter in the kelp *Laminaria sp.,* and subsequently used during spring growth (Mann 1982, Raven et al. 1992). Although ammonium is not regarded as a major nitrogen storage compound in macroalgae, intracellular ammonium concentrations have been observed to increase following uptake (Naldi and Wheeler 1999). To a larger extent, organic forms of nitrogen are stored in algae where uptake exceeds growth (Gagne et al 1982, Raven 1987b, Raven et al. 1992, Naldi and Wheeler 1999). These reserves are typically reduced when growth rates exceed nitrogen uptake (Syrett 1981, Mann 1982).

Seasonally, macroalgal productivity may be limited by light availability and temperature (Norin and Waern 1973, Valiela et al. 1997). When light and temperature are sufficient for maximum growth rates, typically during spring and summer, increases in algal growth rates tend to be limited by the availability of nutrients (Rhee 1980, Hanisak 1983, Fujita et al. 1989, Peckol et al. 1994, Valiela et al. 1997).

The relationship between growth and nutrient availability in algae can most simply be explained by models such as that developed by Monod (1942) for growth of microbes limited by a single substrate. In these, the creation of new biomass is linked simply to the uptake rate of the substrate. However in macroalgae, where limiting nutrients are

stored in intercellular pools, growth is often decoupled from external nutrient supply, and instead can be related to the intracellular concentration of the limiting nutrient (Rhee 1980). In this case, growth rates relative to internal nutrient supply typically follow a rectangular parabola, with an asymptote equal to the maximum specific growth rate (Rhee 1980), and drop sharply as the internal concentrations of the limiting nutrient approach zero.

Macroalgae have varying maximum nitrogen uptake rates, and specific growth rates which roughly follow morphological lines. Algae with smaller surface area to volume ratios, such as kelps, tend to have slower maximum N uptake and growth rates while frondose algae with higher surface area to volume ratios tend to have higher uptake and growth rates (Wallentinus 1984). More frondose algae also tend to have higher nitrogen requirements. This is in part due to their generally higher tissue nitrogen concentrations, but also due to the demand for nitrogen required for the creation of new tissue (Pedersen & Borum 1997). In ecosystems where all growth requirements are available in excess, a small number of fast growing taxa with high uptake and growth rates will tend to dominate, displacing slower growing macrophytes and reducing diversity (Lavery et al. 1991, Peckol et al. 1994, Valiela et al. 1997).

The movement of nutrients and energy through ecosystems is difficult to measure directly. It is possible to measure the uptake and storage of nitrogenous compounds by marine primary producers in field situations, but this offers no direct information on the sources of the nutrients available for uptake. With knowledge of both source and volume of nitrogen available to marine primary producers, managers can take action to remedy sources of nitrogen that may be problematic to marine systems. When

measuring nutrient movement through food webs, gut content analysis can provide details on the amount of food consumed, but this is labour-intensive, difficult in small organisms, and can be misleading where consumption volumes differ from nutritional values (Smit 2001).

Stable isotopes facilitate indirect measurement of nutrient pathways through food chains. The approach relies upon isotopic "signatures" to infer trophic linkages in complex ecosystems (Fry and Sherr 1984). The approach also facilitates tracing and quantifying sources of terrestrially derived enrichment, pollution and sediments that may enter aquatic environments (delGiorgio and France 1996, McClelland and Valiela 1998b, Jones et al. 2001, Bouillon et al. 2002, Gordon and Goni 2003).

This approach uses the differences in the background levels of isotopes in components of the biosphere to determine patterns of nutrient transfer. Stable isotopes of most ecologically relevant elements are made up of one extremely abundant isotope, and one or more rare isotopes. Where natural differences exist in the levels of enrichment in pools of biologically relevant elements, their relative enrichment levels can be used to trace the movement of these pools in space and through food chains. The rarity of the secondary isotopes also allows for the use of artificially enriched sources of the rare isotope as tracers in environmental and physiological studies. In some cases, stable isotope analysis offers the only feasible tool to study the subtle impacts of different nitrogen and carbon sources on marine ecosystems.

Differences in the atomic mass of the isotopes of an element can result in different chemical behaviour of one isotope relative to another. The lighter isotope (with a

lower atomic mass) typically forms bonds that can be broken more easily. This difference in chemical behaviour can cause different isotopes to vary in their representation in reactant and product pools of a reaction. (i.e., the lighter isotope generally becomes more concentrated in the product relative to the heavier isotope). As elements pass through chemical reactions in different pools in biological systems, this process of 'kinetic fractionation' leads to isotopes existing in different quantities in each pool. The main uses of stable isotopes in ecology (e.g., for nutrient source identification and trophic level analysis) rely upon: 1) predictability in fractionation processes as pools of isotopes are taken up, (e.g., uptake of nitrogen and carbon by algae, and during nitrogen and carbon transfer along food chains); and 2) the stability and consistency of the 'signatures' of possible nutrient sources.

There is considerable evidence to suggest that these assumptions are rarely met in studies of natural systems (Gannes et al. 1997). Notably, carbon and nitrogen stable isotope ratios in primary producers have been shown to vary independently of source $\delta^{13}C$ and $\delta^{15}N$ as a function of the physical and chemical conditions during growth (Raven et al. 2002, Finlay 2004, Needoba et al. 2004). Furthermore, the transfer of stable isotope signatures along food chains has been shown to be inconsistent between species (Gannes et al. 1997, Vanderklift and Ponsard 2003, Fry 2006). For example, fractionation during nitrogen assimilation has been suggested to be greater in herbivorous than in carnivorous fish due in part to increased excretion rates in herbivores (Mill et al. 2007). Finally, $\delta^{13}C$ and $\delta^{15}N$ signatures of inorganic carbon and nitrogen sources available to marine producers are also variable in time and space (Smit 2001, Bedard-Haughn et al. 2003). Thus, successful design and interpretation of ecological and environmental stable isotope studies require a system-specific

understanding of the reliability of isotope ratio transfer, and the physiological processes that cause variability in the isotope signatures of source pools.

Background, Stable Isotopes as tracers in ecological and environmental studies

Concepts, measurement and terminology

Ecological studies that employ stable isotope ratios use either variation in naturally occurring levels ('natural abundance' stable isotope studies), or add and follow the path of artificially enriched substances in a system ('enriched' studies).

In this study, I examine only naturally occurring levels of stable isotopes of carbon and nitrogen. The standard measure of carbon (^{12}C to ^{13}C) and nitrogen (^{14}N to ^{15}N) natural isotopic abundances are as delta (δ) ratios in parts per thousand (‰). These ratios are a measure of the level of presence of the heavier isotope relative to its level of presence in an internationally accepted reference standard (see table 1.1). The equations for calculating δ ratios for carbon and nitrogen are:

$$\delta^{13}C = 1000.\left[\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{PDB}}\right] \text{ and } \quad \delta^{15}N = 1000.\left[\frac{^{15}N/^{14}N_{sample}}{^{15}N/^{14}N_{air}}\right]$$

Where the term '*sample*' refers to the sample being analysed, '*PDB*' is a reference standard of carbonate from the cretaceous PDB formation, and '*air*' is atmospheric nitrogen. Henceforth, references to a sample or pool of nitrogen or carbon as 'heavier'

indicates a relative enrichment in the heavier isotope (either ^{13}C or ^{15}N), and 'lighter' indicates a sample less enriched in the heavier isotope.

Table 1.1. Carbon and nitrogen, their isotopes, their percent abundance in nature, their reference standard and percent abundance of isotopes in that standard. (Modified from Dawson et. al. 2002)

Element	**Isotope**	**Percent natural abundance**	**Standard**	**Abundance ratio of reference standard**
Nitrogen	^{14}N	99.63	Atmospheric N_2	3.6764×10^{-3}
	^{15}N	0.3663		
Carbon	^{12}C	98.982	PDB carbonate	1.1237×10^{-2}
	^{13}C	1.108		

Stable isotope levels are measured using mass spectrometry. Advances in instrumentation over the last 25 years, particularly the development of automated systems for carbon and nitrogen analysis, have contributed to growth in the number of stable isotope studies conducted as part of ecological research. All analysis for this study was performed using an automated, continuous flow isotope ratio mass spectrometer (CF-IRMS). More information on isotope ratio measurement and instrumentation may be found in a review by Dawson and Brooks (2001).

Variation in natural abundance stable isotope ratios of inorganic source N and C pools in seawater

Nitrogen

Dissolved inorganic forms of nitrogen (DIN) in marine and estuarine systems form the majority of the N supply that sustains primary production in these environments (DeBoer 1981). $\delta^{15}N$ ratios of DIN in marine systems tend to vary in space and time, as a function of the $\delta^{15}N$ ratios of the pools of DIN entering the system, and

fractionation effects within the system (Smit 2001, Fry 2006). Within system fractionation effects that alter DIN $\delta^{15}N$ ratios include sedimentary processes of biological nitrification and oxidation, as well as decomposition and mineralization of organic N (Bedard-Haughn et al. 2003, Fry 2006). The movement of external sources of DIN into marine systems can also create gradients of isotope ratios in primary producers, and these gradients can be passed upwards through food chains (Spies et. al 1989, McClelland and Valiela 1998, Hadwen and Arthington 2007).

Some $\delta^{15}N$ values of nitrogen potentially available for uptake for marine primary producers are listed in table 1.2. Data for this table are sourced from reviews by Peterson (1999), Smit (2001), Bedard-Haughn et al. (2003), Fry (2006) and studies by Rogers (1999, 2003), Gartner et al. (2002), Savage and Elmgren (2004) and this study.

Table 1.2 $\delta^{15}N$ ratios of some potential DIN sources for primary producers.

N species	Source	Mean (‰)	Range (‰)
Ammonium	Remineralised (estuarine)	13.0	10 to 16
DIN total	Fertiliser	0	-2 to 2
N_2 (air)	atmosphere	0	
Nitrate	North Pacific	6.1	
Nitrate	Groundwater	6 to 8	0 to 20
Total N	Septic tank	10	8 to16
Total N	Animal urine	-8	-3 to -12
DIN total	Tertiary processed sewage effluent	~20	7 to 38
DIN total	Primary or non processed sewage effluent	2	0 to 5

In some cases the disparate $\delta^{15}N$ values of these sources of nitrogen are the result of fractionation in reactions that occur prior to their movement into marine systems. For example, volatilisation of ammonium during processing of sewage prior to release can increase the $\delta^{15}N$ ratio of the dissolved nitrogen remaining in the effluent (Bedard-Haughn et al. 2003, Savage and Elmgren 2004). The more rapid loss of ^{14}N than ^{15}N during decomposition of particulate N pools results in a 5‰ -10‰ increase in $\delta^{15}N$ ratios with increasing depth in the ocean and in soils (Fry 2006). Notably, the disparity of $\delta^{15}N$ ratios in N pools within the biosphere is limited because biological processes such as photoautotrophic growth are often limited by the availability of nitrogen. Where this is the case, all (or most) nitrogen is likely to be assimilated, regardless of isotope content (Fry 2006). For details on causes of significant fractionation in terrestrial pools of nitrogen that may be available to marine systems, see reviews by Bedard-Haughn et al. (2003) and Fry (2006).

Carbon

Bicarbonate ions (HCO_3^-) and dissolved carbon dioxide ($CO_{2(aq)}$) in equilibrium make up more than 99% of the dissolved inorganic carbon (DIC) in the world's oceans. Fractionation in the equilibrium reaction between the two compounds results in the $\delta^{13}C$ of HCO_3^- being around 11‰ higher than that of $CO_{2(aq)}$ (Raven et al. 2002). As a result, variability in the $\delta^{13}C$ of source carbon available to photoautotrophs may come about as a result of changes to the chemical equilibrium of DIC in marine waters. Significant inter-species differences in marine primary producer tissue $\delta^{13}C$ ratios are also generated by tendencies for some species to assimilate only $CO_{2(aq)}$ (particularly some of the Rhodophyta) and others to assimilate a mixture of $CO_{2(aq)}$ and HCO_3^-

(Raven et al. 2002). Some intertidal species may also assimilate atmospheric $CO_{2(aq)}$ (with a $\delta^{13}C$ of -7.8 ppt) when exposed at low tide (Surif and Raven 1990).

Methods and assumptions for calculation of isotopic mixing

Studies that use the C and/or N isotopes to estimate nutrient contributions of a range of possible sources normally employ mixing models (e.g., Ben-David et al. 1997, Ben-David and Schell 2001, Phillips et al. 2005). Much of the discussion in the following chapters refers to the assumptions of these models.

The simplest of these models is a two-end mixing model e.g. Spies et al. (1989). Where two possible nutrient sources exist, an estimate of the percentage contribution of each source to the organism's nitrogen content can be generated using a two-end mixing model, shown here using the example of an effluent nitrogen contribution to the total nitrogen budget of an alga.

$$\delta^{15}N_x = X(\delta^{15}N_{effluent}) + (1-X)(\delta^{15}N_y)$$

Where X is the proportion of effluent contribution, $\delta^{15}N_x$ is the $\delta^{15}N$ of the impacted alga, $\delta^{15}N_y$ is the background $\delta^{15}N$ value. Model from Spies et al. (1989), modified by Wayland and Hobson (2001).

Studies that estimate dietary contributions often employ mixing models that attempt to distinguish the relative contributions of more than two possible food sources (Ben-David et al. 1997, Ben-David and Schell 2001, Phillips et al. 2005). An example of a model that uses both $\delta^{13}C$ and $\delta^{15}N$ ratios to distinguish contributions of three possible food sources is shown below (modified from Smit (2001)).

The food source values for A, B and C in the equations below are typically adjusted to account for fractionation. This adjustment can be made using standard figures for fractionation factors between source and consumer (e.g. Smit (2001)), but can be adjusted to accommodate variable fractionation, (see Vanderklift and Ponsard 2003, Sweeting et al. 2007).

This method first calculates Euclidean distances between the delta ratios of each possible food source and the consumer (equation 1 below, calculates one food source), and then calculates the contribution of each food source to the consumer's diet as the inverse of the Euclidean distance between the source and the consumer (equation 2 below)

1. $$Z_A = \sqrt{\delta^{13}C_A - \delta^{13}C_{consumer})^2 + (\delta^{15}N_A - \delta^{15}N_{consumer})^2}$$

Where Z_A is the Euclidean distance between the consumer and food source 'A', and $\delta^{13}C_A$ and $\delta^{15}N_A$ are the signatures for food source 'A'.

2. $$\%X = \frac{Zx}{Z_A^{-1} + Z_B^{-1} + Z_C^{-1}} \times 100$$

Where Z_X is the Euclidean distance between any of the food sources and the consumer, and Z_A^{-1} is the inverse of the Euclidean distance of food source 'A' from the consumer.

There are several key assumptions that are important to the success and validity of these models.

1. That fractionation is known for each instance of nutrient transfer.
2. That the food sources are significantly separated in their $\delta^{13}C$ and $\delta^{15}N$ ratios.

3. That the mean isotope ratios of potential nutrient sources used in the analysis are accurate over the temporal and the spatial scale of the study.

Aims

This thesis is intended to add to our body of knowledge of the processes of carbon and nitrogen isotope assimilation in marine algae, and the effects of these processes on techniques that employ carbon and nitrogen stable isotope ratios in marine ecology. The model system for this work is the flow of carbon and nitrogen from a sewage outflow in Titahi Bay, New Zealand to a shallow, rocky reef. Using field studies in this system and laboratory experiments, these aims are addressed in the following manner:

1. Examining the stability of $\delta^{15}N$ levels in *Ulva* sp. in reflecting ^{15}N-enrichment from sewage effluent loading under varying conditions of light and nitrogen availability.
2. Examining the stability of tissue $\delta^{13}C$ in *Ulva* sp. (i.e. a carbon source at the base of a marine food chain) over gradients of light and nutrient availability, such as can be found at many terrestrial/marine interfaces.
3. Investigating the reduction of nitrate to ammonium during nitrogen uptake in *Ulva* sp. as a possible mechanism for growth inhibition and increased fractionation in *Ulva* sp. under light-limited conditions.
4. Investigating patterns of variability in sewage effluent $\delta^{15}N$ and $\delta^{13}C$ source signatures between treatment type and processing facilities.

5. As a case study, using the differences in $\delta^{15}N$ and $\delta^{13}C$ of sewage and natural marine sources to illustrate feeding patterns of marine consumers under sewage plume influence.

In chapter 2, I use a logistic model to examine the effect of depth (as a proxy for light availability) in *Ulva* sp. transplanted to sites in a gradient away from the Titahi Bay Wastewater Treatment Plant (TBWWTP). I then examine the effect of light availability and nutrient source (nitrate or ammonium) on *Ulva* sp. tissue $\delta^{15}N$ signatures experimentally in controlled conditions. I experimentally examine growth-rate driven change in *Ulva* sp. tissue $\delta^{13}C$ ratios under controlled conditions with nutrient and light limited growth. I compare these results with patterns of tissue $\delta^{13}C$ measured in *Ulva* sp. transplanted to sites in a gradient away from the TBWWTP. In chapter three, I address the third aim above using a series of controlled experiments with manipulations of nitrogen source and light availability. In chapter four I address the fifth aim of this thesis by tracing the movement of $\delta^{13}C$ and $\delta^{15}N$ signatures in source pools of sewage carbon and nitrogen measured at the TBWWTP into producers and consumers in an impacted community. Using analysis of covariance (ANCOVA), I assess patterns of $\delta^{13}C$ and $\delta^{15}N$ signatures in a macroalga, a filter feeder and an herbivorous grazer in relation to patterns of dispersal of particulate and dissolved effluent fractions. Appendix 5 contains an assessment of variability in $\delta^{13}C$ and $\delta^{15}N$ in signatures of dissolved and particulate components of sewage effluent following two common treatment types at three treatment plants.

Chapter 2 – Effects of light variation and nutrient addition on the $\delta^{15}N$ and $\delta^{13}C$ ratios of *Ulva* sp.

Abstract

The use of stable isotope signatures to infer sources of enrichment of nitrogen and carbon to ecological systems relies on predictability in the expression of the source $\delta^{15}N$ and $\delta^{13}C$ signatures by primary producers. In this study, assimilation rates of ^{14}N vs ^{15}N and ^{13}C vs ^{14}C under varying light conditions and nitrogen (N) source (e.g., nitrate or ammonium) are examined for macroalgae.

To examine nitrogen and carbon fractionation in *Ulva* sp., I manipulated light availability and N source (either nitrate, ammonium or no nutrient addition) in factorial designs in laboratory experiments. My results suggest that: 1) low-light conditions can produce algae with lower $\delta^{15}N$ and $\delta^{13}C$ signatures; 2) for $\delta^{15}N$, this effect was more pronounced for ammonium than for nitrate sources. 3) $\delta^{13}C$ ratios were dependent on sufficient supply of nutrients (nitrogen and phosphorus) as well as light levels during growth. In laboratory experiments, *Ulva* sp. tissue $\delta^{13}C$ ratios ranged from -5.99‰, (grown for 14 days with ambient light and 10 μM added ammonium) to -17.6‰, (grown for 14 days in ambient light, with no added nutrients). Fractionation rates during assimilation of ammonium by *Ulva* sp. were separated by 3.7‰ between high light and low light treatments.

In a complimentary field study I transplanted individuals of *Ulva* sp. in a distance gradient away from a point source sewage discharge, near the seawater surface and at 4 m depth. Field measurements of seawater salinity, nitrogen and phosphorus ions, and *Ulva* sp. $\delta^{15}N$ ratios indicated that ^{15}N-enriched sewage effluent moved in a

buoyant plume; however *Ulva* sp. grown at depth had comparatively lighter $\delta^{15}N$ signatures than surface sites estimated to receive the same concentrations of sewage effluent. Stable carbon isotope ratios ($\delta^{13}C$) of *Ulva* sp. covaried spatially with levels of dissolved sewage in surface waters. These results provide a cautionary message for some applications of stable isotope studies, and generally underscore the need for an improved mechanistic understanding of stable isotope assimilation into organisms and ecological systems. These results may be particularly important for ecological stable isotope studies conducted in areas where light and nutrient availability vary over short spatial scales, such as at the marine/freshwater boundary.

Introduction

The application of carbon and nitrogen isotope analysis in the study of marine food web linkages and movement of terrestrial matter into the marine environment has become commonplace over the last 30 years. The utility of the techniques lies in the tendency of carbon and nitrogen pools to retain their $\delta^{13}C$ and $\delta^{15}N$ ratios to some extent as they pass through ecological systems. For example, $\delta^{13}C$ ratios of animal tissues tend to reflect those of their food sources with only minor, predictable changes (Fry 1984). Many of these studies use distinct isotopic signatures of marine plants and algae to determine feeding patterns of consumers and the distribution of terrestrial carbon and nitrogen sources (Ben-David and Schell 2001, Dunton 2001, Usui et al. 2006, Fry 2006, Catenazzi 2007). The use of marine algae in ecological stable isotope studies relies on an understanding of how isotope signatures of algal tissue reflect inorganic source carbon and nitrogen isotope signatures, and how this varies under varying physical conditions.

Nitrogen enrichment to aquatic ecosystems is a widespread consequence of human population growth. Common sources of enrichment of aquatic and coastal environments include sewage discharge and agricultural runoff, which can cause increases in the growth rates of nitrogen and phosphorus limited plants and algae (Campbell 2001, Armitage et al. 2005). Coastal enrichment can also alter biotic interactions (Bokn et al. 2002, Hauxwell et al. 2003, Karez et al. 2004, Tewfik et al. 2005) and patterns of biodiversity (Morris 1991). Worldwide, there is increased pressure to regulate nitrogen enrichment; efficient and reliable tools to identify and quantify sources of nitrogen enrichment are required.

Natural abundance nitrogen stable isotope signatures in macroalgae ($\delta^{15}N$ signatures) are increasingly used as a tool to trace sources of nitrogen enrichment across a wide range of ecological systems (Aravena et al. 1993, Erskine et al. 1998, McClelland and Valiela 1998, Lojen et al. 2005, Kaushal et al. 2006). This technique has been used with success to estimate the effect of terrestrially derived nitrogen on marine systems (McClelland et al. 1997, McClelland and Valiela 1998, Rogers 1999, Costanzo et al. 2001, Gartner et al. 2002, Savage and Elmgren 2004, Costanzo et al. 2005, Savage et al. 2004, Cornelisen et al. 2007). The accuracy of some inferences drawn from this approach however, depend upon a number of assumptions, which commonly remain untested.

Nitrogen stable isotopes are commonly viewed as useful tracers of enrichment because different sources of nitrogen often have characteristic and widely divergent signatures. For example, dissolved inorganic nitrogen (DIN) from unprocessed

sewage typically has a $\delta^{15}N$ value of approximately 5-6‰ (McClelland and Valiela 1998, this study), and these signatures typically become progressively "heavier" (i.e., enriched with ^{15}N relative to ^{14}N) due to greater volatilisation of ^{14}N than ^{15}N during treatment prior to discharge (e.g., reported values of processed sewage range from 7 to 38‰ (Savage et al. 2004). DIN in groundwater entering coastal watersheds from natural soils typically has less enriched $\delta^{15}N$ values, between 2 and 8‰ (Macko and Ostrom 1994).

The accuracy of $\delta^{15}N$ signatures as tracers of enrichment sources hinges on primary producers incorporating the $\delta^{15}N$ signature from the environment to their own tissue in a predictable manner. Tracer signatures are often evaluated indirectly from organisms (e.g., from primary producers, as opposed to directly from seawater) because such organisms facilitate a time-integrated estimate of exposure. Specifically, this approach requires that any discrimination between ^{14}N and ^{15}N that is exercised by a primary producer as it incorporates and retains nitrogen within its tissue remains consistent over the spatial extent of the study.

For marine plants and algae, this process of discrimination between ^{14}N and ^{15}N that results in a difference between the $\delta^{15}N$ signature of inorganic nitrogen in seawater, and the $\delta^{15}N$ signature of the algae (i.e. 'fractionation') may occur at any of several stages. Discrimination during incorporation of DIN from seawater into algal tissues may occur during active uptake of DIN over the plasma membrane, and/or during reduction of NO_3^- to NO_2^- and subsequently to NH_4^+ (catalysed by nitrate reductase and nitrite reductase respectively), and finally during synthesis of amino acids and subsequent compounds (Needoba et al. 2004). Typically, the heavier isotopes (which

form slightly stronger chemical bonds) are less represented in the product pools of these processes. Notably, for discrimination that takes place within the algal cell to result in a fractionation effect between seawater DIN and algal tissue, some of the isotopically heavier reactant pools need to be effused from the plant.

Previous work suggests that light availability (Wada and Hattori 1978, Heikoop et al. 1998, Needoba and Harrison 2004), growth rate (McKee et al. 2002), nutrient availability (Waser et al. 1998b, McKee et al. 2002), turbulence (Neill Barr, personal communication) and N source (Waser et al. 1998a) may all have some effect on fractionation during nitrogen incorporation in photoautotrophs.

The $\delta^{13}C$ isotopic signatures of photoautotrophs, including marine algae, are a function of the isotopic signature of the inorganic carbon available for uptake, and their tendency to assimilate ^{12}C into their tissue at a different rate than ^{13}C. This discrimination in the assimilation of carbon isotopes causes differences between the $\delta^{13}C$ ratio of inorganic source carbon (e.g. seawater dissolved inorganic carbon (DIC)) and that in algal tissue.

The size of the difference between the $\delta^{13}C$ signatures of source carbon and the $\delta^{13}C$ of algal tissue depends primarily on the magnitude and expression of isotope effects during photosynthesis (Farquhar et al. 1982, Raven and Farquhar 1990, Maberly et al. 1992, Raven et al. 2002). Additionally, as inorganic carbon in seawater is composed of HCO_3^- and $CO_{2(aq)}$ in equilibrium, and these two sources of carbon possess distinct $\delta^{13}C$ signatures in seawater, the $\delta^{13}C$ of algal tissue depends of the proportion of the two ions taken up and assimilated (Raven et al. 2002).

The expression of isotope effects varies between algal species, as a result of physiological differences in carbon assimilation (Laws et al. 1997, Raven et al. 2002). The two main sources of isotope effects during carbon assimilation arise during uptake of carbon across the plasma membrane (Farquhar et al. 1989, Raven and Farquhar 1990) and in carbon fixation. The enzymes that facilitate carboxylation, (one of the first major processes in the Calvin cycle) discriminate against the heavier isotope (^{13}C). The enzyme RuBisCO is responsible for around 95% of CO_2 fixation in plants, and acts more rapidly with ^{12}C than ^{13}C, in effect a ~30‰ discrimination against the heavier isotope for dissolved CO_2 (Farquhar et al. 1989, Raven and Farquhar 1990). This results in an enrichment of the $\delta^{13}C$ of intracellular CO_2 relative to that in seawater. An expression of this isotope effect as a difference between the $\delta^{13}C$ of algal tissue and that of seawater (fractionation) relies on leakage of the ^{13}C-enriched internal CO_2 back out of the alga. A large leakage relative to the rate of carbon assimilation will lead to a large fractionation effect, a small leakage will lead to a small fractionation effect (Farquhar et al. 1989). Inter-species differences in the $\delta^{13}C$ ratios of algae living in identical physical environments are to a large part caused by differences in this leakage, and in the relative proportions of HCO_3^- and $CO_{2(aq)}$ that each species assimilates (Raven et al. 2002).

In addition to inter-species differences, substantial variation in the expression of isotope effects in tissue $\delta^{13}C$ levels within a species can result from differences in the physical conditions in which algae grow. Factors that reduce the availability of CO_2 for carbon synthesis are likely to reduce the level of fractionation associated with carbon synthesis. At least some species of algae are able to reduce the leakage of CO_2

from the cytosol by increasing the expression of a carbon concentrating mechanism under conditions of low CO_2 availability (Raven 2001).

In phytoplankton, a lower concentration of $CO_{2(aq)}$ at the site of carboxylation relative to photosynthetic demand tends to lead to a higher proportion of ^{13}C being assimilated and a relative increase in the $\delta^{13}C$ ratio of algal tissue (Laws et al. 1995, Laws et al. 2002). Increased growth rates typically increase internal CO_2 demand relative to supply (Fry and Wainright 1991, Laws et al. 1995, Burkhardt et al. 1999b, Laws et al. 2002). Where growth rate is resource-limited, $\delta^{13}C$ ratio tends to be negatively correlated with availability of the limiting resource (Laws et al. 2002).
Where light and temperature are sufficient for maximum growth, the availability of the nutrient in shortest supply relative to demands will tend to limit growth rate in macroalgae (DeBoer 1981). The effects of growth-rate limiting factors on phytoplankton $\delta^{13}C$ ratios have been shown for phosphorus limitation (Gervais and Riebesell 2001), light limitation (Leboulanger et al. 1995, Thompson and Calvert 1995, Riebesell et al. 2000) and nitrogen limitation (Riebesell et al. 2000). In temperate coastal waters growth rates of opportunistic macroalgae are most commonly limited by availability of nitrogen (Twilley 1985, Peckol et al. 1994), particularly seasonally in periods of high light and temperature.

Interpretation of nitrogen and carbon stable isotope data in coastal ecological studies would benefit from a greater understanding of the drivers of variability in the $\delta^{13}C$ and $\delta^{15}N$ signatures of the marine plants and algae that form the base of food chains. This is particularly pertinent to studies where measurements are made over gradients of freshwater and/or anthropogenic influence such as at the freshwater/marine

boundary. In such cases, as well as potential variability in the $\delta^{13}C$ and $\delta^{15}N$ ratios of DIC and DIN, there may be variability in the availability of a suite of factors, such as light and nutrient availability, which may potentially result in changes to the isotope ratios of plants and algae.

Experimental overview

To date, few studies have attempted to quantify nitrogen isotopic fractionation in macroalgae, (Cohen and Fong 2005, Cornelisen 2007). Here I employ field observations of sewage influence on the opportunistic macroalga *Ulva* sp. *in situ*, and the resulting $\delta^{15}N$, $\delta^{13}C$ and tissue N content (%N).

I use controlled laboratory experiments to quantify fractionation patterns of nitrogen and carbon isotopes in *Ulva* sp. under variable depth and nutrient environments. Importantly, my experimental treatments are guided by environmental conditions that I observed at a New Zealand coastal site impacted by a sewage discharge.

Methods

The general approach for this work consisted of a field assessment of a sewage effluent discharge site at Titahi Bay, Wellington, New Zealand (41° 7'S, 174° 49'E), coupled with two controlled laboratory experiments conducted at the Leigh Marine Laboratory, New Zealand (36°16'S, 174°48'E). At Titahi Bay, I sampled seawater and effluent chemistry and plant tissue isotope concentrations at multiple sites and repeated time intervals. Sites were arrayed in a distance gradient east (generally "upcurrent", n=5) and west ("downcurrent", n=5) of the Titahi Bay wastewater treatment plant (TBWWTP); one additional site was located 50 m immediately

offshore of the TBWWTP discharge pipe (Fig 2.1). Sites along this gradient were permanently marked with buoys, and established along subtidal rocky reef habitat, approximately 50 m offshore, in approximately 4 m of water depth, and approximately 100 m apart. During the six month period of my study, TBWWTP released an average of 237.9 litres of tertiary processed sewage per minute (range = 0 - 1048 l/min), with an average DIN concentration of 0.158 mmol (n = 40; S.E. = 0.012). Effluent discharged from TBWWTP is generally tertiary processed, except in cases of extremely high flow, when some overflow passes directly from the primary screening facility to the outflow. Discharge patterns from October 1 2004 to April 1 2005 are given in Figure 2.2.

Field estimates of seawater and effluent chemistry

I used a Niskin bottle to collect seawater samples at approximately monthly intervals (5 dates between November 2004 and March 2005) at the surface and at 4 m depth from each of 11 study sites. Samples were transferred to polycarbonate bottles and stored on ice in the field and subsequently frozen at -20ºC until they could be analysed. Ammonium and phosphate concentrations were estimated following methods of Koroleff (1983), nitrate and nitrite were estimated using methods of Parsons et al. (1984). To characterise spatial and temporal variation in plume concentration, I measured levels of ammonium/ammonia (NH_4^+/NH_3), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^-) in all seawater samples. I used a multivariate analysis of variance (MANOVA) to explore variation in all variables between surface and 4 m depths and among sites. MANOVA results were informally evaluated against expected decreasing concentration gradients with increasing distance from the effluent outflow, and possible differences between concentrations of sewage-derived

nutrients in the eastern and western arms of the mooring array. Results of a Wilks lambda test are given in table 2.1, and because many of the variables appeared to behave similarly and were related to sewage enrichment, I used a principal components analysis (PCA) to construct an aggregate variable to characterize overall patterns of sewage influence. PC1 scores were then regressed against salinity, nitrogen and phosphate ions in seawater samples to test for fit. The first principal component (PC1) from this intermediary analysis explained 68.9% of the variance in the data set, and in the context of the high variability in this system, I deemed this sufficient to justify the use of PC1 scores for each site as a proxy for 'sewage influence' in subsequent statistical models.

Measurements of salinity and temperature were made using an RBR XR420 data logger on 5 runs over 4 dates between January and April 2005. Measurements were made in surface waters (i.e., 0.5 m depth) and at 4 m depth at all sites on all dates. The data logger was programmed to record at 5-second intervals and was deployed in surface water and subsequently at 4 m for a period of 1 minute at each location. Salinity and temperature estimates were generated by time-averaging data acquisitions over the period of deployment (1 minute = 12 data acquisitions).

Figure 2.1

Figure 2.2

To characterise effluent composition that was discharged from TBWWTP, I sampled effluent at weekly intervals (n=3) in March 2005. Samples were always collected at ~9am to control for possible diel variation in sewage composition and/or processing. Effluent samples were refrigerated at 4˚C on site and then frozen within 3 hours of collection until they could be processed. All effluent samples were alkali distilled in the presence of Devarda's alloy to reduce nitrate to ammonium. Total N concentration was determined by back titration with standardised acid. Samples were prepared for mass spectrometry by drying the remaining solution in excess acid.

Field estimates of nitrogen and carbon isotope signatures

To quantify time-integrated measures of spatial variation in carbon and nitrogen isotope signatures over a distance gradient away from the TBWWTP discharge, I sampled tissue composition of a common primary producer, the macroalga *Ulva* sp. All algae used for this assay were collected from a common site in Wellington Harbour (at a location that was not subject to nitrogen enrichment by sewage). Algae were deployed near the surface and at a depth of 4m at each of my sites, and secured by rope weave to the buoy lines marking each of my permanent locations. Algae were deployed in early October 2004, and algal tissues were first sampled in late November 2004. Tissue samples were collected from all algae at ~8 week intervals from November 2004 to March 2005 (n=3). Samples were maintained on ice in the field and subsequently frozen until they could be analysed.

To calculate estimates of carbon and nitrogen isotope signatures within *Ulva* sp., samples were cleaned of any epiphytes and epifauna, dried at 70˚C in a drying oven

and ground to a fine powder. All isotope samples were analysed using a Europa Geo 20/20 isotope ratio mass-spectrometer interfaced to an ANCA-SL elemental analyser. Duplicate samples of 1.8 mg of powder were loaded into tin capsules for analysis of organic carbon and nitrogen content and carbon and nitrogen isotopic composition. The standard analytical error between duplicate analyses is lower than ±0.3‰ for nitrogen and ± 0.1‰ for $\delta^{13}C$. Relative isotopic concentrations are reported as $\delta^{15}N$ values relative to an air standard, where

$$\delta^{15}N‰ = \left(\frac{^{15}N/^{14}N_{sample} - ^{15}N/^{14}N_{air}}{^{15}N/^{14}N_{air}} \right) \times 1000$$

All $\delta^{13}C$ values were normalised to PDB using Europa flour, IAEA Beet sugar and ANU-sucrose where

$$\delta^{13}C‰ = \left(\frac{^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{PDB}}{^{13}C/^{12}C_{PDB}} \right) \times 1000$$

I explored spatial variation in isotope signatures ($\delta^{15}N$ and $\delta^{13}C$) graphically using time-averaged signatures (± S.E.) of plants collected from each site, near the surface and at depth. I examined the manner in which $\delta^{15}N$ signatures in *Ulva* sp. act as indicators of nitrogen enrichment from sewage by regressing spatial variation in $\delta^{15}N$ values (among sites and between depths) on to predicted sewage effluent concentrations.

Because my $\delta^{15}N$ data were predicted to be bounded by asymptotes at 6‰ and 23.4‰ (see below), I used logistic regression bounded by these values in *Ulva* sp. at two depths over a range of sewage effluent concentrations. The model measured sewage

concentration with 'predicted plume concentration' (PC1), treating "depth" (either near surface or at a depth of 4m) as a factor with two levels.

The upper asymptote for the logistic model was calculated from the $\delta^{15}N$ ratio of sewage effluent DIN (23.4‰), based on the assumption that algae receiving 100% sewage effluent would prefer the lighter isotope during uptake and assimilation, if any preference was shown. The lower asymptote was set at 6‰ based on data from *Ulva* sp. grown in environments with little or no influence from anthropogenic sources of nitrogen (Rogers 1999, Gartner et al. 2002).

I evaluated the degree to which *Ulva* sp. $\delta^{13}C$ levels and tissue nitrogen levels in *Ulva* sp. tissues were affected by sewage plume presence and depth using analysis of covariance. ANCOVA models used either *Ulva* sp. $\delta^{13}C$ levels or tissue nitrogen content as the dependent variable, with estimated plume concentration (PC1) as a covariate and depth as a factor with two levels. A third ANCOVA model, to examine patterns of spatial variability in tissue nitrogen not related to effluent dispersal again used depth as a factor, but used distance (covariate), and direction (factor with two levels) in place of PC1 to predict changes in *Ulva* sp. tissue nitrogen content. This analysis initially used a fully crossed model. Interactions that were non significant in all months ('depth x distance' and 'depth x distance x direction') were subsequently removed, and significance tests from the reduced model are presented.

To avoid confounding temporal (e.g., seasonal) variation in plant physiology and/or environmental conditions, I conducted separate analyses for each of the three successive sampling periods (i.e., for plants deployed from mid October to late

November, late November to mid January and mid January to early March). These and all following statistical analyses were performed using the R statistical package (R development core team, 2005).

Performance of $\delta^{15}N$ signatures under experimentally manipulated environmental conditions

Experimental overview

To determine the effect of light reduction and nutrient source on the expression of $\delta^{15}N$ signatures in *Ulva* sp., I conducted an experiment that manipulated light levels and available nitrogen source (either nitrate or ammonium) under controlled conditions in a full factorial design. I measured $\delta^{15}N$ ratios, algal growth rates and tissue nitrogen content of *Ulva* sp. tissue grown in these conditions. The latter measurements enabled me to infer important relationships between environmental conditions, nitrogen demand, and the ecological responses (e.g., growth) of *Ulva* sp. to these conditions.

To determine the effect of light reduction and nutrient availability on the expression of $\delta^{13}C$ signatures in *Ulva* sp., I conducted a separate experiment that manipulated light levels and nutrient availability (either 10 µM of added ammonium or no nutrient addition) under controlled conditions in a full factorial design.

Outdoor chemostat setup

General apparatus

I used an outdoor seaweed on-growing apparatus for maintaining *Ulva* sp. in turbulent conditions under different light and nutrient regimes. The design of individual seaweed growth chambers is shown in Fig. 2.3. Each container had a liquid volume of 4.5 L determined by the height of its drain weir. A turbulent seawater flow design was incorporated into the growth chambers using pivoted 'dump buckets' at one end of each growth chamber. Each dump bucket tipped 0.75 L of water into the growth chamber when full.

Natural low-nutrient seawater was coarsely filtered (200 μm) to remove any large objects that could cause blockages in the apparatus, and supplied continuously to the experimental apparatus via a nutrient scrubber (see below). A constant supply of seawater to the on-growing apparatus was maintained using a header tank.

The culturing system comprised two rows of 8 growth chambers. Each row of eight chambers was supplied with seawater directly from the header tank via a supply manifold at a rate of 1.2 l min^{-1} per chamber (via the dump bucket).

Figure 2.3

Nutrient scrubber

During the course of the $\delta^{15}N$ experiment, filtered coastal seawater was supplied to the chemostat via a nutrient scrubber, designed to remove nitrogen in seawater prior to the additions (with known $\delta^{15}N$ ratios) supplied to the experimental system. Seawater was run through a longitudinal race (approximately 9 meters long by 200 mm wide and 100 mm deep) containing free-floating *Ulva* sp. plants. Seawater was pumped through the system at 25 l min^{-1}. The nutrient stripped seawater was then pumped to a header tank of the system as described above with surplus seawater fed back to the start of the nutrient scrubber. Levels of ammonium and nitrate in header tank water, monitored throughout the $\delta^{15}N$ experiment, were (mean ± S.E) 0.1 μM ± 0.1 and 0.1 μM ± 0.01 respectively. Ammonium measurements in seawater entering and exiting the algal nutrient scrubber are displayed in Figure 2.4. The data displayed were collated from measurements made on a single day (n=3) and analysed using a deionised water blank. During the course of the $\delta^{13}C$ experiment, filtered coastal seawater was supplied directly to the chemostat header tank.

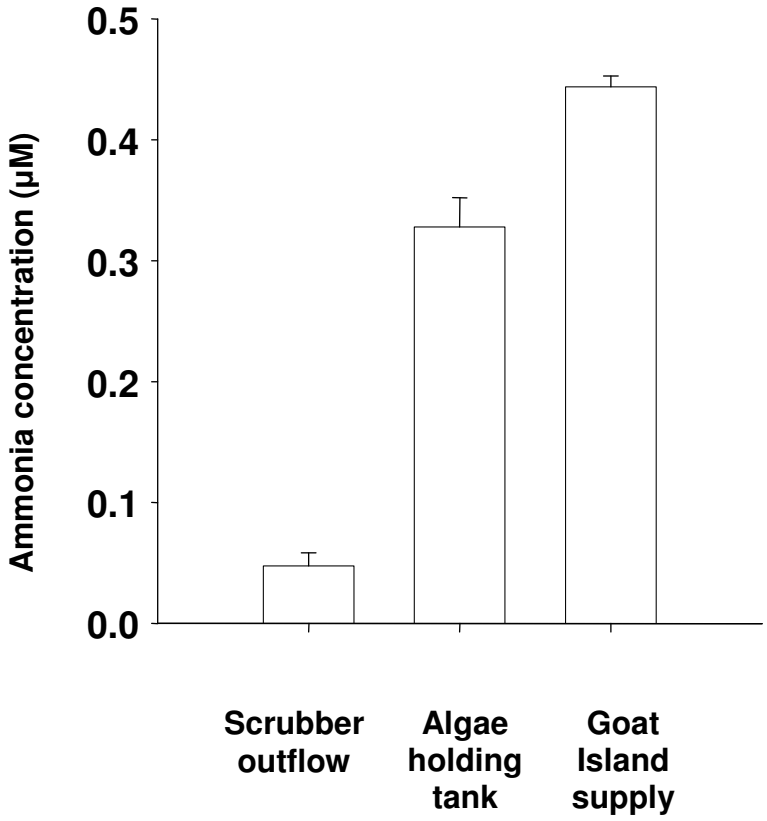

Figure 2.4. Ammonium reduction effects of the algal nitrogen scrubber. 'Goat Island supply' refers to samples of seawater taken from the laboratory inflow. Around 50% of the water entering the scrubber flowed from a seaweed holding tank (i.e. 'Algae holding tank'). Water from the scrubber outflow was supplied to the ODC header tank for the $\delta^{15}N$ experiment.

Nutrient treatment

Nutrient treatments provided a standardized dose of ~ 10 µM of available nitrogen and 2 µM of phosphate to algae for both ammonium and nitrate treatments, and this dose was selected because it approximated the level of nitrogen enrichment recorded near the TBWWTP. Solutions containing an appropriate combination of ammonium chloride, sodium nitrate or dihydrogen phosphate were continuously added into each dump bucket at a rate of 1.75 ml h^{-1} using a 16-channel peristaltic pump to provide a steady concentration of available nitrogen and phosphorus. Stock solutions were individually made and supplied to each of the pump's 16 channels. The concentration of stock solution varied for each replicate depending on the measured rate of seawater input for that replicate. Final seawater nutrient concentrations in each replicate were

checked and adjustments were made as necessary to either the pump flow rate or the individual stock concentrations.

Light treatment

'Shaded' treatment chambers were covered by 3 layers of 50% neutral density screen (a.k.a. mosquito mesh) giving an irradiance extinction coefficient of around 82%. Light extinction measurements were made underwater at the surface of the thalli using a Biospherical scalar irradiance (photosynthetic active radiation [PAR]) probe, model QSL2100.

Light levels at a weather station at the Leigh Marine laboratory were recorded over the duration of the experiments.

Acclimation of plant tissue to artificial $\delta^{15}N$ signatures prior to $\delta^{15}N$ experimental trial

Specimens of *Ulva* sp. were collected from a common location in Tauranga harbour (37º 39'S, 176º 11'E). During storage, algae were provided with a constant flow of filtered coastal seawater from the laboratory's seawater flow-through system and maintained at 50% ambient (outdoor) light levels and at ambient temperature. Freshly harvested tissue contained nitrogen with a different $\delta^{15}N$ to that used in the experiment. In order to attribute differences in isotope signature between light and shade treatments at the end of the experimental trial to fractionation it was necessary to turn over all algal tissue using artificial nitrogen as an N source. Otherwise,

differences in growth and N uptake rates between treatments would affect the proportion of nitrogen in plant tissue derived from oceanic or artificial sources.

Prior to initiating the $\delta^{15}N$ experimental trial, plants were grown in the experimental chambers for 2 weeks on nitrogen of known $\delta^{15}N$ signatures added to the growth chambers, and ambient light levels. In order to remove tissue not equilibrated to the artificial nutrient sources and ambient light conditions, algal masses in each chamber were measured every two days during this period and approximately half of the algal tissue mass was discarded at each weighing to return the experimental biomass to around 3g. This procedure was repeated 8 times during the preparation period until > 99% of the nitrogen in the *Ulva* sp. tissue was from 'artificial' added nitrogen. The constant supply of 'artificial' $NaNO_3$ or NH_4Cl provided during preparation came from the same chemical batches used as nitrogen supply during the experimental period. $\delta^{15}N$ signatures of the batches of $NaNO_3$ and NH_4Cl used were measured in triplicate prior to lab trials. All algal tissue was prepared outdoors under ambient light.

Experimental design

Algae were grown under experimental conditions for a period of 14 days. I manipulated nutrient source (either ammonium or nitrate) and light availability (either shaded or ambient (outside in full sun)) in a fully crossed design. I included four replicates for each treatment (a total of 16 separate chambers), and positions of treatments within the chemostat were randomized. Sea surface temperatures at the extraction point of the laboratory seawater flow through system were recorded daily.

I quantified tissue nitrogen content $\delta^{13}C$ and $\delta^{15}N$ ratios for *Ulva* sp. samples from each experimental chamber, using methods described for field assay samples (above). To estimate growth rates, I weighed *Ulva* sp. specimens on 8 occasions over 14 days. Prior to weighing, excess moisture was removed using a salad drier. After each weighing, tissue mass of all specimens was standardized to 3g by trimming.

Algal growth rates in lab experiments were calculated for each time interval using a logistic growth equation:

$$W_t = W_0 \times e^{\mu t}$$

where W_t is the weight of algae at time t, W_0 the initial weight and μ the specific growth rate (d^{-1}).

For $\delta^{15}N$ I calculated 'fractionation' during nitrogen assimilation as the difference between $\delta^{15}N$ ratios calculated for *Ulva* sp. tissue and for added NH_4Cl and $NaNO_3$. For $\delta^{13}C$ where the available inorganic carbon source was identical for each treatment, I did not calculate fractionation, but compared final (day 14) $\delta^{13}C$ ratios between treatments.

Statistical analysis

I used two-way analysis of variance (ANOVA) to test for differences in tissue $\delta^{15}N$ and $\delta^{13}C$ values resulting from experimental manipulations of nutrients and/or light. Posterior pairwise comparisons between combinations of treatments were made using Tukey's HSD test to adjust for multiple comparisons. An analogous two-way ANOVA was used to test effects of experimental treatments on tissue nitrogen

content. To test the effect of treatments on growth rates of *Ulva* sp., I used analysis of variance of mean growth rates over the duration of the experiment.
Data were tested for normality and homogeneity of variance, and post-hoc comparisons were performed using Tukey's HSD test, which adjusts significance statistics for multiple testing. All analyses were performed using the R statistics package. (R Development Core Team, 2005).

Results

Variation in sewage effluent constituents

MANOVA performed on the responses of all measured sewage constituents suggests sewage effluent concentrations varied significantly as a function of depth, direction, and distance from the sewage outfall (Table 2.1). A significant interaction between 'depth' and 'distance' effects was also detected (i.e., the disparity between samples from surface and deeper waters attenuated with distance from the outfall). Generally, sewage constituents (e.g., nitrate, ammonium, and phosphate) were elevated in surface waters close to the outflow, consistent with an expected concentration gradient of treated sewage constituents. Concentrations of nitrite were comparatively low (< 1µM) throughout sampled waters, and did not appear to vary systematically in relation to the sewage outflow.

The primary constituents of sewage effluent that I quantified show evidence of a westerly advection away from the point source of discharge, and this appears to be consistent with mean flow patterns in the region (personal observations), and patterns

of salinity (Fig. 2.5). PC1 scores, when regressed against the primary constituents of sewage effluent most closely tracked concentrations of nitrate, and TIN measured in field seawater sampling (Figure 2.6).

Salinity measurements on all dates, sites and depths ranged between 34.89‰ and 33.52‰. Seawater temperatures on all dates, sites and depths ranged between 19.7 ºC and 16.5 ºC. On average, surface sites were 0.057 ºC (± 0.017 S.E. n = 55) warmer than 4 m sites.

Table 2.1. Results of MANOVA analysis examining trends in dispersal of the Titahi Bay WWTP effluent plume

	df	Wilks	Approximate F	Numerator d.f.	Denominator d.f.	p
Depth	1	0.84	4.74	4	102	0.002
Direction	1	0.63	14.91	4	102	<0.001
Distance	1	0.90	2.78	4	102	0.030
Depth*Distance	1	0.87	3.89	4	102	0.006
Residuals	105					

Analyses of samples of tertiary-processed sewage effluent collected directly from TBWWTP indicate values (mean, S.E.) for constituents that are as follows: ammonium (35.56 µM, ± 8.42), nitrate (120.69 µM, ± 8.77), nitrite (1.72 µM, ± 0.39), TIN (157.97 µM, ± 12.46), $\delta^{15}N$ signature of DIN for unprocessed effluent 5.5 ± 0.1, $\delta^{15}N$ signature of DIN for tertiary processed effluent 23.43 ± 2.14.

Variation in $\delta^{15}N$ and $\delta^{13}C$ signatures recorded in a primary producer

Nitrogen stable isotope signatures in *Ulva* sp. individuals retrieved from moorings at all sites, depths and months ranged between 6.33 ‰ and 22.53 ‰. Carbon stable isotope signatures in *Ulva* sp. individuals retrieved from moorings at all sites, depths and months ranged between -22.2 ‰ and -13.1 ‰.

Time-averaged $\delta^{15}N$ and $\delta^{13}C$ ratios recorded within tissue of *Ulva* sp. samples varied in pattern spatially (among sites and between depths) in a way that is consistent with spatial variation in sewage effluent constituents (compare Fig. 2.5 with Fig. 2.7). Because I suspected that the "performance" of $\delta^{15}N$ as a predictor of sewage constituents might differ qualitatively between *Ulva* sp. tissue sampled near the surface and at depth, I tested this hypothesis explicitly using logistic regression. Overall, these models suggest that for a given level of available sewage constituents, *Ulva* sp. tissue sampled from 4m depth generally had significantly lower $\delta^{15}N$ ratios relative to *Ulva* sp. tissue collected from surface waters (Table 2.2, Fig. 2.8). While the relationship between PC1 and $\delta^{13}C$ ratios appeared to be more distinct at surface sites (Fig. 2.9), there was no overall evidence of an influence of depth on $\delta^{13}C$ ratios (Fig. 2.7, Table 2.3A).

Figure 2.5

Figure 2.6

Figure 2.7

Figure 2.8

Figure 2.9

Table 2.2. ANOVA comparisons of logistic model fit describing the covariance of *Ulva* sp. $\delta^{15}N$ ratios with sewage influence (PC1). Model 1 does not include depth as a factor; model 2 includes depth as a factor.

Period	Model	Residual *df*	Residual SS	*df*	SS	F	p
Oct -Nov	1	15	0.27				
	2	17	0.23	2	0.04	1.42	0.273
Nov- Jan	1	15	0.60				
	2	13	0.37	2	0.23	4.06	0.043
Jan-Mar	1	14	0.08				
	2	12	0.05	2	0.03	4.08	0.044

Table 2.3A. Results of separate ANCOVAs testing for the influence of sewage influence and depth on $\delta^{13}C$ ratio of *Ulva* sp.

Model : response = PC1 + depth + (PC1 x depth)

Response factor	Source of variation	*df*	SS	MS	F	p
$\delta^{13}C$ November	PC1	1	8.525	8.525	2.961	0.1024
	depth	1	5.174	5.174	1.797	0.1967
	PC1 x depth	1	4.071	4.071	1.414	0.2498
	Residuals	18	51.82	2.879		
$\delta^{13}C$ January	PC1	1	23.16	23.16	14.5122	0.0015
	depth	1	0.6820	0.6820	0.4274	0.5225
	PC1 x depth	1	0.0657	0.0657	0.0412	0.8417
	Residuals	16	25.53	1.596		
$\delta^{13}C$ March	PC1	1	6.041	6.041	12.24	0.0035
	depth	1	0.5435	0.5435	1.1014	0.3117
	PC1 x depth	1	0.1046	0.1046	0.2119	0.6523
	Residuals	14	6.908	0.4934		

Table 2.3B. Results of separate ANCOVAs testing for the influence of sewage influence and depth on tissue nitrogen content of *Ulva* sp.
Model : response = PC1 + depth + (PC1 x depth)

%N November	PC1	1	1.405	1.405	6.127	0.0235
	depth	1	1.994	1.994	8.696	0.0086
	PC1 x depth	1	1.379	1.379	6.016	0.0246
	Residuals	18	4.127	0.2293		
%N January	PC1	1	2.550	2.550	6.882	0.0184
	depth	1	1.276	1.276	3.444	0.0820
	PC1 x depth	1	0.0165	0.0165	0.0444	0.8357
	Residuals	16	5.930	0.3706		
%N March	PC1	1	0.1295	0.1295	0.4563	0.5103
	depth	1	0.0845	0.0845	0.2979	0.5938
	PC1 x depth	1	0.4401	0.4401	1.551	0.2334
	Residuals	14	3.972	0.2837		

Because I suspected that heavier *Ulva* sp. $\delta^{13}C$ ratios in surface moored algae may have been due to nitrogen-driven increases in growth rates, I examined *Ulva* sp. tissue nitrogen content in relation to sewage influence and depth. ANCOVA results showed significant increases in tissue nitrogen content of *Ulva* sp. at sites of higher sewage influence (Table 2.3B). This effect was not consistent over all time periods. Tissue nitrogen levels of algae sampled in March were highly enriched at all sites, and no significant patterns were apparent. Additionally, time averaged tissue nitrogen content data showed spatial patterns of nitrogen enrichment that could not be explained solely

by PC1 or a PC1 by depth interaction (Fig. 2.10). A separate ANCOVA analysis of this pattern showed a tendency for algae to the west (more sewage influenced) to be enriched in nitrogen at surface sites, while algae at sites to the east were similarly enriched at 4 m sites and depleted at surface sites (Table 2.4 (November), Fig. 2.7 and Fig. 2.10). Overall, while *Ulva* sp. $\delta^{13}C$ ratios and tissue nitrogen content followed sewage influence (PC1) with some consistency, tissue nitrogen levels also appeared to vary spatially in patterns that could not be explained fully by sewage influence (Fig. 2.9 and 2.10). Linear regression of *Ulva* sp. $\delta^{13}C$ levels against tissue nitrogen content suggests a relationship between these two tissue indices, particularly at surface (high light, high plume influence) sites (Fig. 2.11).

Figure 2.10

Fig. 2.11

Table 2.4. Results of ANCOVAs testing for the influence of distance and direction from the TBWWTP, and depth, on tissue nitrogen in *Ulva* sp.
Model : response = distance + direction + depth + (direction x depth)

Response factor	Source of variation	*df*	SS	MS	F	p
Log %N November	distance	1	0.0234	0.0234	0.0841	0.7753
	direction	1	0.4608	0.4608	1.6551	0.2155
	depth	1	0.5317	0.5317	1.9097	0.1849
	Direction x depth	1	3.157	3.157	11.34	0.0037
	Residuals	17	4.733	0.2784		
%N January	distance	1	0.0001	0.0001	0.0004	0.9848
	direction	1	1.626	1.626	5.439	0.0340
	depth	1	2.949	2.949	9.8662	0.0067
	Direction x depth	1	0.7144	0.7144	2.390	0.1429
	Residuals	15	4.484	0.2989		
%N March	distance	1	0.0033	0.0033	0.0115	0.9163
	direction	1	0.3609	0.3609	1.253	0.2832
	depth	1	0.1682	0.1682	0.5841	0.4583
	Direction x depth	1	0.3506	0.3506	1.218	0.2898
	Residuals	13	3.743	0.2879		

Light, temperature and nutrient conditions during laboratory experiments

Daily solar radiation levels at the Leigh Marine Laboratory station during the $\delta^{13}C$ experiment varied between 32.44 MJm^{-2} (Day 2), and 19.46 MJm^{-2} (day 11). The mean daily radiation over the duration of the experiment was 27.48 MJm^{-2} ±1.22 S.E (n = 14). Sea surface temperature at the laboratory inflow point ranged between 17.0 ºC (day 2) and 20.3 ºC (day 13). Mean sea surface temperature over the duration of the experiment was 18.7 ºC ± 0.25 S.E (n = 14). Mean concentrations of nitrate, ammonium and phosphate in high light, nutrient-added treatments were 0.14 ± 0.09 µM, 12.85 ± 0.18 µM and 1.38 ± 0.04 µM respectively. Mean concentrations of nitrate, ammonium and phosphate in low light nutrient-added treatments were 0.12 ± 0.14 µM, 12.20 ± 0.21 µM and 1.26 ± 0.01 µM respectively. Mean concentrations of nitrate and ammonium in header tank water were 0.13 ± 0.10 µM and 0.0 ± 0.04 µM.

Daily solar radiation levels at the Leigh Marine Laboratory station during the $\delta^{15}N$ experiment varied between 29.11 MJm^{-2} (day 7), and 7.07 MJm^{-2} (day 3). The mean daily radiation over the duration of the experiment was 21.37 $MJm^{-.2}$ ± 1.38 S.E (n = 15). Sea surface temperature ranged between 20.3 ºC (day 14) and 21.1 ºC (day 5). Mean sea surface temperature over the duration of the experiment was 20.6 ºC ± 0.06 S.E (n = 15). Mean concentrations of nitrate, ammonium and phosphate in high light nutrient-added treatments were 10.56 ± 0.23 µM, 10.54 ± 0.15 µM and 1.94 ± 0.03 µM respectively. Mean concentrations of nitrate, ammonium and phosphate in low light nutrient-added treatments 9.97 ± 0.22 µM, 9.45 ± 0.09 µM and 1.97 ± 0.07 µM

respectively. Mean concentrations of nitrate and ammonium in header tank water were 0.1 ± 0.01 and 0.1 ± 0.1 µM.

Laboratory experiment 1 - Performance of $\delta^{15}N$, tissue nitrogen content and algal growth under experimentally manipulated environmental conditions

Differences in $\delta^{15}N$ ratios between source nitrogen and *Ulva* sp. tissue (fractionation) were significantly ($p < 0.001$) higher in low light treatments (Fig. 2.12, Table 2.5). This increase corresponded to higher tissue nitrogen content and lower growth rates for both nutrient treatments receiving low light treatment (Fig. 2.12 and 2.14, Table 2.5). A significant interaction between factors 'light treatment' and 'nutrient treatment' indicates that the effect of light reduction on fractionation during growth is dissimilar for nitrate and ammonium. A post-hoc Tukey's paired analysis comparing the effect of light reduction on fractionation between ammonium and nitrate substrates suggests a strong relationship between light reduction and fractionation for ammonium-fed plants ($p < 0.001$), and a weak relationship between light reduction and fractionation during growth on nitrate ($p = 0.062$).

Laboratory experiment 2 - Performance of $\delta^{13}C$ signatures tissue nitrogen content and algal growth under experimentally manipulated environmental conditions

Laboratory trials using nitrogen concentrations comparable to those found in high impact and low impact sites in field sampling would indicate that the large differences in $\delta^{13}C$ signatures found in field sampling could be generated by differences in

nutrient supply during periods of high growth of this species. After 14 days of growth, *Ulva* sp. supplied with seawater with added nutrients showed significantly higher $\delta^{13}C$ signatures than those supplied with unmodified coastal seawater ($p < 0.001$, Fig. 2.13, Table 2.6). There was a significant interaction in the effect of light and nutrient treatments on $\delta^{13}C$ levels ($p < 0.001$). Ambient light treated algae also receiving nutrients showed the highest $\delta^{13}C$ values (mean $\delta^{13}C$ -5.99‰), while ambient light treated algae not receiving nutrients generated the lowest $\delta^{13}C$ values (mean $\delta^{13}C$ -17.61‰). Shade treated algae receiving ambient and nitrogen added seawater averaged final $\delta^{13}C$ levels of -13.70‰ and -10.20‰ respectively.

Due to the nature of this interaction there was overall no significant light effect, because the response to light treatment depended on whether the algae were receiving nutrients or not. A post-hoc Tukey's paired analysis suggests all combinations of treatments shown in Fig. 2.13 are significantly separated with $p < 0.001$.

Patterns of higher $\delta^{13}C$ ratios in the tissue of algae in added nutrient treatments, particularly in high light, correspond with patterns of increased tissue nitrogen content and growth rates of these treatments (Fig. 2.13 and 2.14, Table 2.6).

Plants treated with added ammonia and shade showed final tissue nitrogen concentrations between 3 and 3.5%, close to the maximum value encountered in field plants, while the 'light control' treatment gave a mean tissue nitrogen percentage below 1, slightly below the minimum single value encountered in field plants.

Fig. 2.12

Figure 2.13

Figure 2.14

Table 2.5. Results of two way ANOVAs testing for the influence of nutrient treatment and light treatment on $^{14}N/^{15}N$ fractionation, tissue nitrogen content and growth rates in experiment 1.

Response factor	Source of variation	*df*	SS	MS	F	p
Fractionation	Nutrient	1	0.39	0.39	2.23	0.16
	Light	1	20.51	20.51	117.44	<0.001
	Nutrient*light	1	8.14	8.14	46.60	<0.001
	Residuals	12	2.10	0.17		
%N	Nutrient	1	0.01	0.0008	0.03	0.86
	Light	1	4.21	4.21	180.02	<0.001
	Nutrient*light	1	0.01	0.01	0.37	0.56
	Residuals	12	0.29	0.02		
Growth rate	Nutrient	1	0.0001	0.0001	0.1255	0.7293
	Light	1	0.0406	0.0406	99.33	<0.001
	Nutrient*light	1	0.000008	0.000008	0.0198	0.8905
	Residuals	12	0.0049	0.0004		

Table 2.6. Results of two way ANOVAs testing for the influence of nutrient treatment and light treatment on $\delta^{13}C$, tissue nitrogen and growth rates in experiment 2.

Response factor	Source of variation	*df*	SS	MS	F	p
$\delta^{13}C$	light	1	0.067	0.067	0.4363	0.5275
	Nutrient	1	171.46	171.46	1108.3	<0.001
	Light x Nutrient	1	49.41	49.41	319.39	<0.001
	Residuals	8	1.238	0.155		
%N	light	1	0.073	0.073	128.8	<0.001
	Nutrient	1	0.621	0.621	1098.1	<0.001
	Light x Nutrient	1	0.022	0.022	39.11	<0.001
	Residuals	8	0.0045	0.0006		
Growth rate	light	1	0.0846	0.0846	147.85	<0.001
	Nutrient	1	0.1647	0.1647	93.15	<0.001
	Light x Nutrient	1	0.0315	0.0315	17.85	0.0029
	Residuals	8	0.0141	0.0018		

Discussion

Despite the growing application of stable isotope tracers in ecological studies, there has been relatively little consideration of the mechanisms—physiological and/or environmental—that underlie the incorporation of these signals into ecological systems. This is particularly in the case of $\delta^{15}N$ ratios that are commonly evaluated and interpreted from marine and freshwater algae and plants. I was able to locate very little information evaluating or even describing predictability of ^{15}N behaviour under varying environmental conditions and levels of nutrient availability. (However see Cohen and Fong 2005, Cornelisen 2007.) Incorporation of $\delta^{15}N$ ratios within microalgae from aquatic environments is known to vary among species, and as functions of temperature, light, and nutrient availability (Wada and Hattori 1978, Waser et al. 1998a, Waser et al. 1998b, Waser et al. 1999, Needoba et al. 2003, Needoba and Harrison 2004), and consequently, observed signatures are not necessarily a simple reflection of $\delta^{15}N$ ratios found in a particular nitrogen source. There is considerable information to suggest that macroalgal $\delta^{13}C$ values vary spatially (Cloern et al. 2002, Raven et al. 2002, Vizzini et al. 2005) and temporally (Cloern et al. 2002, Vizzini and Mazzola 2003) within species in marine systems. There is also considerable information to suggest that $\delta^{13}C$ values of photoautotrophs, both marine and terrestrial, vary in response to environmental variables that affect the physiology associated with growth and carbon assimilation (Dawson et al. 2002, McKee et al. 2002, Raven et al. 2002). There is, however, a paucity of information regarding seasonal and spatial patterns of macroalgal $\delta^{13}C$ values as a function of environmental variables. This information is required for ecologists to better design studies of carbon transfer in coastal marine systems. Here, my results clearly show how light availability alters observed stable isotope signals, and moreover, that for

$\delta^{15}N$ ratios this effect differs qualitatively among different forms of nitrogen, and for $\delta^{13}C$ ratios this varies on the concentration of nitrogen available for uptake. My laboratory results show how $\delta^{15}N$ ratios respond to alterations in light levels, and the source of inorganic nitrogen available for uptake (nitrate or ammonium). These results also show how $\delta^{13}C$ values measured in *Ulva* sp. respond to variations in light and nutrient availability. I recorded significant variation (11.6‰) in $\delta^{13}C$ values in algae grown for 14 days in controlled conditions. This variation can be attributed to an increase of 10μM in the concentration of available dissolved nitrogen, and the interaction between light levels and nitrogen concentration. I note that this range in $\delta^{13}C$ values extends over the natural range of a considerable number of macroalgal species (i.e. possible food sources for marine consumers (Raven et al. 2002)). Natural recorded ranges of *Ulva* sp. from published literature are shown in table 2.7.

Table 2.7. Ranges of published $\delta^{13}C$ ratios from genus *Ulva* $\delta^{13}C$ ratios including maximum and minimum ratios from experimentally manipulated and field conditions in this study.

Species	Notes	Origin	Date collected	$\delta^{13}C$ ratio (‰)	Reference
Ulva lactuca	Drift	Eden estuary, Scotland, UK	8 January 1995	-11.05; -11.35	Raven et al. 2002
Ulva lactuca	Attached	Brighton Beach/Papatowai Beach, New Zealand	1 February 1996	-15.5	Raven et al. 2002
Ulva lactuca	-	Brighton Beach, South of Dunedin, New Zealand	1 July 1998	-17.65	Raven et al. 2002
Ulva sp.	-	Gran Canaria	1 February 1991	-15.64	Raven et al. 2002
Ulva sp.	-	Hampton Bay, Long Island, NY, USA	16 November 1994	-16.79	Raven et al. 2002
Ulva sp.	-	Singapore	1 April 1996	-17.72	Raven et al. 2002
Ulva sp.	-	Helmsdale, Scotland, UK	26 July 1998	-15.91	Raven et al. 2002
Ulva lactuca	Attached –High sewage impact site	Wellington, New Zealand	October 1997 – February 1998	- 13.3	Rogers 2003
Ulva lactuca	Attached – Low sewage impact site	Wellington, New Zealand	October 1997 – February 1998	-17.1	Rogers 2003
Ulva sp.	Beach cast	Paracas Bay, Ica Region,Peru	January –April 2003	~ -10.3 – - 12	Catenazzi and Donnolly 2007
Ulva pertusa	Attached –South facing (low irradiance)	Doubtful sound, New Zealand	July 2004	-18.2	Cornelisen et al. 2007
Ulva pertusa	Attached – North facing (high irradiance) site	Doubtful sound, New Zealand	January 2004	- 12.0	Cornelisen et al. 2007
Ulva sp.	Transplanted – Surface moored, high sewage impact site	Titahi Bay, Wellington, New Zealand	January 2005	-13.1	This study
Ulva sp.	Transplanted – Moored at 4 m subsurface, low sewage impact site	Titahi Bay, Wellington, New Zealand	March 2005	-22.2	This study
Ulva sp.	High light, Low N coastal seawater (Nitrogen starved)	Laboratory grown	January 2006	- 17.61	This study
Ulva sp.	Low light, Low N coastal seawater	Laboratory grown	January 2006	- 13.70	This study
Ulva sp.	High light, 10 µM ammonium and 1 µM phosphate added to coastal seawater.	Laboratory grown	January 2006	- 5.99	This study
Ulva sp.	Low light, 10 µM ammonium and 1 µM phosphate added to coastal seawater.	Laboratory grown	January 2006	- 10.20	This study

Ecological implications

One general consequence of my findings concerns the application of $\delta^{15}N$ ratios to infer patterns of eutrophication. Values of $\delta^{15}N$ in study organisms are often evaluated over environmental gradients that can include factors that may co-vary with concentrations of enriched nitrogen. As a result, interpretation of patterns of nutrient enrichment may be confounded by environmental gradients (e.g., light or nutrient type) that affect the transfer of $\delta^{15}N$ in organisms such as *Ulva* sp. Similarly, the widespread use of stable isotopes to infer trophic pathways may be suspect under some environmental conditions (e.g., in regions of varying light, and nitrogen availability, such as found in marine and freshwater mixing points). In my laboratory experiment, I observed significant variation (3.7 ‰) in $\delta^{15}N$ ratios for a given concentration of nutrients. My experimental design enables me to attribute this variability to the form of the nitrogen source (either ammonium or nitrate), light availability, and the interaction between these two environmental variables. I note that the range of variability observed among my experimental treatments that received a constant concentration of nitrogen enrichment is similar to the ranges of $\delta^{15}N$ values in many well-designed environmental studies used to infer nutrient concentration gradients (e.g., McClelland and Valiela 1998, Gartner et al. 2002).

To illustrate the potential effect of fractionation on estimates of sewage nitrogen contribution to *Ulva* sp. tissue we can use a 2-end mixing model from Spies et al. (1989), (modified by Wayland and Hobson (2001)).

$$\delta^{15}N_x = X(\delta^{15}N_{effluent}) + (1-X)(\delta^{15}N_y)$$

Where X is the proportion of effluent contribution, $\delta^{15}N_x$ is the $\delta^{15}N$ of the impacted alga, and $\delta^{15}N_y$ is the background $\delta^{15}N$ value. We will use the example of time averaged $\delta^{15}N$ signatures from surface site W3 (18.65‰), and assume a constant sewage DIN $\delta^{15}N$ signature of 23.4‰ and a background $\delta^{15}N$ signature of 6.1‰ (the second figure based on $\delta^{15}N$ values of oceanic nitrate from Bedard-Haughn et al. 2003). If we also assume that mean fractionation during nitrogen assimilation at this surface site is zero, we can calculate that sewage contributed around 72.5% of the nitrogen assimilated by *Ulva* sp. algae at this site. In this case, net discrimination of 2.0 ‰ for the lighter isotope during algal assimilation of nitrogen in seawater would cause a shift in this estimate to 61%.

Isotopic mixing models, which are employed to calculate the relative contributions of carbon to diets and ecosystems, generally use a mean $\delta^{13}C$ value for each potential source of carbon (Smit 2001, Gordon and Goni 2003, Usui et al. 2006). Where these carbon sources are assimilated into consumers or sediments, the accuracy of the models which calculate the relative contributions of sources rely on the stability (or at least predictability) of individual source $\delta^{13}C$ values over the space and time that they have been integrated (Ben-David et al. 1997, Smit 2001). A further consequence of my findings concerns $\delta^{13}C$ dietary studies that extend across spatial gradients of light and nitrogen availability. Where variability in light and nutrient levels are high, between-species differences in primary producer $\delta^{13}C$ values may be obscured by spatial variability in primary producer $\delta^{13}C$ values. For more robust food web and trophic level studies I suggest that consideration be given to the effects of spatial patterns of nutrient limitation on algal $\delta^{13}C$ values. Additionally I suggest studies that include macroalgae as a potential food source compile their mean values of algal $\delta^{13}C$

over spatial scales that represent the possible foraging areas of consumers included in the study to accommodate spatial variability in nitrogen and light availability.

A second implication of these results relates to the tracing of terrestrial sediments. Terrestrial plant matter has a tendency towards lighter (more negative) $\delta^{13}C$ signatures than marine carbon, and retains this signature when it is transported into the marine environment (Gordon and Goni 2003). This tendency can be used to trace the origin of organic carbon in marine sediments (Gordon and Goni 2003, Usui et al. 2006). A heavier algal $\delta^{13}C$ signature driven by the presence of nutrient rich terrestrial waters, such as that seen in the field portion of this study, could potentially lead towards an isotopically heavier, more 'marine' signature in sediments.

For robust ecological interpretations of nitrogen stable isotopes, I suggest that more attention to the mechanisms of their uptake and assimilation into organisms and ecological systems may be needed. Certainly, I would advocate that the assumptions of predictability of incorporation of $\delta^{15}N$ signatures into primary producers ought to be tested or at least considered more frequently.

Further research

My results and those of others (e.g., Needoba et al. 2004) suggest some fruitful avenues for future exploration into some of the mechanisms that may underlie nitrogen stable isotope uptake. In particular, my observations suggest that ^{15}N fractionation in primary producers varies as a function of light and growth. I suggest that such patterns may be the result of differences in the availability of nitrogen relative to its rate of uptake and assimilation by algae. Previous studies show that

while N fractionation in higher plants and aquatic microalgae tends to vary between species (Montoya and McCarthy 1995, Waser et al. 1998b, Needoba et al. 2003, Pritchard and Guy 2005), different species may exhibit similar patterns in fractionation response to changing physical conditions and available nitrogen concentrations (Waser et al. 1999, Kolb and Evans 2003). This suggests that my findings and inferences about how nitrogen availability may influence fractionation within *Ulva* sp. may extend more broadly to other species of macroalgae.

In this study, patterns of nitrogen fractionation observed under high light/rapid growth and low light/slower growth, with ammonium as a nitrogen source, are consistent with studies on microalgae and bacteria in batch cultures with similar treatments (Wada and Hattori 1978, Hoch et al. 1992, Hoch et al. 1994, Montoya and McCarthy 1995, Waser et al. 1998a, Waser et al. 1998b, Waser et al. 1999, Kolb and Evans 2003). However, the maximum fractionation effects observed for *Ulva* sp. in this study, while potentially ecologically significant (with a maximum range of 3.7 ‰ between ammonium light and shade treatments), are low in comparison with some of the above studies.

Despite similar growth rates for algae receiving nitrate and ammonium within light treatments (see Figure 2.14), fractionation evidenced in this study for nitrate was lower than fractionation with respect to source ammonium. Differences between the $\delta^{15}N$ of nitrate-supplied *Ulva* sp. tissue and the $\delta^{15}N$ of the added nitrate-N were -0.19‰ and 0.65‰ for light and shade treatments respectively. The low fractionation of nitrate-supplied algae in both shaded and full-light treatments may indicate that the mechanism of fractionation during growth on nitrate shown in phytoplankton

(Needoba et al. 2004) is not as influential on macroalgal $\delta^{15}N$ signatures. Notably this mechanism relies on the efflux of ^{15}N-enriched internal pools of unreduced nitrate back into the external medium. The capacity of *Ulva* sp. to store nitrate in unreduced form (Naldi and Wheeler 1999) may result in low efflux of nitrate from *Ulva* sp. tissue even when nitrogen is supplied in excess to growth requirements as in the shaded nitrate treatments in this study.

Because fractionation is likely to occur as a result of chemical processes involved in nitrogen assimilation prior to protein synthesis (Needoba et al.2004) it is likely that at least some of the fractionation shown during growth of *Ulva* sp. on ammonium in low light conditions is the result of isotope effects during its diffusion across the plasma membrane. This fractionation does not appear to be as great in the active transport of nitrate across the plasma membrane and its reduction to ammonium under the same environmental conditions.

The apparent positive discrimination for ^{15}N in the ambient light treatment for ammonium is also within the range of previous studies conducted on higher plants and microalgae (Wada and Hattori 1978, Evans et al. 1996, Pennock et al. 1996, Waser et al. 1998a). However, previous evidence of negative fractionation has been attributed to discrimination against ^{15}N during leakage of NH_3 from N-sufficient cells (Waser et al. 1998a). Only net fractionation was measured in this study, and thus no measures of uptake and efflux are available. Further studies are required to isolate the relative contribution of the physiological processes of nitrogen assimilation to the observed patterns of fractionation for nitrate and ammonium in this study.

The low $\delta^{13}C$ of high light, low nutrient *Ulva* sp. tissue compared to that of low light low nutrient treatments also warrants further examination. This pattern suggests that changes in the factor that limits growth (in this case either light or nutrient limitation) may alter the expression of $^{13}C/^{12}C$ fractionation (Riebesell et al. 2000).

The results of this study and of other studies that show seasonal patterns of algal isotope ratios (e.g. Cloern et al. 2002, Vizzini and Mazzola 2003) suggest a need for further research into the seasonal effects of nutrient limitation and growth on macroalgal $\delta^{13}C$ values. This line of research is particularly important in the light of recent research suggesting seasonality in the degree to which consumer tissues reflect food source isotope signatures (Perga and Gerdeaux 2005). In a case where consumer tissues only reflect their food sources in periods of high consumer growth, and those food sources vary seasonally, consumer tissue would become removed from a direct representation of the $\delta^{13}C$ values of the food source they consumed.

Conclusions

Systematic variation in $\delta^{15}N$ signatures arising from light intensity is of a magnitude that necessitates care in the design of studies making use of ^{15}N signatures to infer sources of nutrient enrichment. In addition, effects of light availability on fractionation in *Ulva* sp. are further mediated by the source of nitrogen available to the plant, with ammonium showing a greater tendency towards differential fractionation as a function of light intensity. My work helps to illuminate some of the physiological mechanisms that may be important in fractionation of nitrogen during its assimilation into macroalgae, and has strong implications for a wide range of stable

isotope approaches that rely on understanding of uptake of nitrogen signatures to interpret nutrient and/or trophic pathways through ecological systems.

Variation in this study in $\delta^{13}C$ values in the opportunistic macroalga *Ulva* sp. resulting from nitrogen enrichment and variation in light levels is of a magnitude that suggests care is necessary in the design of stable isotope studies of patterns of carbon flow in coastal marine systems. The correspondence of increased $\delta^{13}C$ values with increased growth rates in experimental trials suggest that like phytoplankton, macroalgae are likely to vary over spatial and temporal gradients of physical variables that influence growth rate.

Chapter 3 – A comparison of growth rates in *Ulva* sp. using nitrate and ammonium as nitrogen sources

Abstract

Increased nitrogen loading to coastal marine systems often results in excessive seasonal growth of marine photoautotrophs, and constitutes an anthropogenic impact of growing global concern. This study examines the growth response of a species of macroalga (*Ulva* sp.) to surplus nitrate and ammonium (two common forms of dissolved inorganic nitrogen available in seawater), under low light and ambient light conditions. I manipulated light and nutrient type (in a factorial design) and used repeated measures ANOVA to infer that *Ulva* sp. experienced a temporary reduction in growth rate and nitrogen assimilation capacity when grown on nitrate, relative to ammonium. This effect appeared to subside after 14 days of growth. The initial spike in growth (and subsequent attenuating response) was mirrored by a temporary reduction in levels of glutamine and free amino acid (FAA) content of *Ulva* tissue. The initially slower response of algal growth rates and nitrogen assimilation of nitrate-fed algae suggests that growth rates of natural populations of *Ulva* may respond more slowly to episodic pulses of nitrate than to ammonium. However, the temporary nature of this effect indicates that growth rates in steady-state populations receiving different proportions of their nitrogen supply via continuous enrichment from nitrate and/or ammonium sources are likely to be similar.

Introduction

Nitrogen is commonly recognised as a primary limiting nutrient to the growth of seaweeds in coastal environments (Hanisak 1983, Smith 1984, Valiela et al. 1997). For many seaweeds, growth during the winter months is further restricted by conditions of low light and low water temperatures. Consequently, growth and performance of macroalgae during certain times of the year may be variably limited by light and/or nutrients (Valiela et al. 1997).

The excess supply of enriching nutrients in coastal marine communities commonly occurs as a result of anthropogenic activities (such as fertiliser additions to pasture, and faecal waste from humans and livestock; GESAMP 2001).

The ecological effects of excess algal growth in coastal marine ecosystems are well documented (Campbell 2001, Bokn et al. 2002, Hauxwell et al. 2003, Karez et al. 2004). Typically nitrogen enrichment results in excessive growth of some macroalgae and phytoplankton during summer months. The high potential growth rates and rapid nitrogen uptake of opportunistic macroalgae give them the tendency to 'bloom' in optimal conditions (Smith et al. 2005). Increases in growth of benthic algae and planktonic photoautotrophs in an ecosystem typically results in a reduction in species richness and an increase in biomass of a small number of species. However, the ecology of this problem is perhaps better understood than the physiology behind the process. Without a solid knowledge of the processes that limit growth in algae, potential managerial responses to excess algal growth in coastal environments are limited.

Of particular interest are the physiological processes that may influence temporal and spatial variation in growth rates of opportunistic algae, and how these may depend upon sources of nitrogen. Because of their potential for rapid growth, 'opportunistic' algae can cause nuisance blooms when all nutritive requirements for growth are supplied in excess (Wallentinus 1984). The availability of nitrogen to natural algal populations tends to be irregular (Sharp 1983). Algae with high surface area to volume ratios such as *Ulva*, which have low storage capacity for nitrogen but high potential nitrogen uptake rates, are well suited to transferring episodic increases in nutrient concentrations into large increases in growth rates in optimal light and temperature conditions (Gagne et al. 1982, Wallentinus 1984). In New Zealand waters, opportunistic green macroalgae of the genus *Ulva* cause nuisance blooms, particularly during spring and summer (Snow 1995).

Nitrogen is available to marine algae in a number of chemical forms. Of these, nitrate and ammonium make up the majority of available nitrogen in coastal waters, while macroalgae are also capable of taking up small quantities of nitrite, urea and organic forms of N (Sharp 1983, Smith et al. 2005). The relative proportions of nitrate and ammonium available to algae varies temporally and spatially, and as a function of the type of anthropogenic activities that may enrich a natural system (Sharp 1983). Algae and aquatic plants typically take up ammonium preferentially to nitrate (Thomas and Harrison 1987), and at faster rates (Wallentinus 1984, Thomas and Harrison 1985). Also, in order for nitrate to be assimilated it is necessary that it first be reduced to ammonium (Syrett 1981, Syrett 1989) (Fig. 3.1). There are several potential costs to growth associated with this reduction. Firstly, there is a greater cost in photons per

unit carbon assimilated during growth on nitrate than would be the case for ammonium (Van Oorschot 1955, Raven 1985). Secondly, there are higher costs of iron, manganese and molybdenum, metals associated with the catalysis of this reduction (Raven 1990). Thirdly, where a significant amount of enzyme is required in order to reduce nitrate, cell doubling times are likely to increase, as more of the product of this reduction (ammonium) is required to produce more enzyme for the same reduction reaction in new tissue (Raven 1984, 1987a).

When the growth of an alga is light limited (energy limited), it may be more susceptible to energetic costs associated with other physiological processes. In this case it is likely that the increase in photons required per unit carbon assimilated should impact on the total carbon produced, and hence the growth rate of the plant. In practice this theoretical cost of reducing nitrate to ammonium is often (De Boer et al. 1978, Layzell et al. 1985), but not always (Mohanty et al. 1991), reflected in a reduction in growth rate. In the case of *Ulva* in coastal waters, lack of trace metals for nitrogen reducing catalysts is unlikely to affect growth rate. Growth in photoautotrophs resulting in coastal waters is unlikely to be limited by trace metal supply, where nitrogen and phosphorus are generally in shorter supply (Hanisak 1983, Valiela et al. 1997).

To date there is conflicting evidence of the advantages to macroalgal growth rates of ammonium rather than nitrate as a nitrogen source. However, some empirical evidence indicates that algae in low light environments are restricted in their capacity to reduce nitrate and instead store it as

Figure 3.1

unreduced KNO_3 (Raven 1987b; Raven 1991), while plants in high light environments store their winter-acquired nitrite in organic reduced form as free amino acids (Raven and Farquhar 1990, Raven 1991). This study employs semi-controlled full factorial laboratory experiments to quantify the nitrate reduction capabilities and growth cost of nitrate reduction by the green macroalga *Ulva* sp. under variable light and nutrient environments.

Methods

Experimental overview

To determine the relative cost of nitrate and ammonium as nitrogen sources for *Ulva* sp., I conducted a series of factorial experiments that manipulated light levels and available nitrogen source under controlled conditions. These experiments were undertaken during summer 2004/5 (experiment 1) and summer 2005/2006 (experiments 2 and 3) at the Leigh Marine Laboratory (36°16'S, 174°48'E). Experiment 1 examined the impact of nutrient source (either nitrate or ammonium), and light levels on growth rates, tissue nitrogen content and free amino acid content of *Ulva* sp. tissue. The latter measurements enabled me to infer relationships between experimental treatments and the ability of *Ulva* sp. to assimilate nitrogen under these conditions. Experiment 2 mirrored the design of experiment 1, with greater frequency of growth measurements to examine the change in effect of nutrient and light treatments over time. Experiment 3 was conducted to mirror experiment 2, but used algal tissue already acclimatised to the experimental treatments, to test if the patterns

shown in experiments 1 and 2 represented an initial acclimatisation of *Ulva* sp. tissue to the experimental treatments.

Specimens of *Ulva* sp. used for all experiments were collected at a common location in Tauranga harbour (37º 39'S, 176º 11'E). Algae were stored prior to use in experiments by providing them with filtered coastal seawater from the laboratory's seawater flow-through system. Under these conditions they were maintained at 50% ambient light levels and at ambient temperature. *Ulva* sp. tissue for experiments 1 and 2 was taken directly from the storage facilities. Tissue for experiment 3 was acclimatised to its experimental nutrient treatment for 14 days prior to the experiment in 50% ambient light levels. 'Nitrate' and 'Ammonium' treatments consisted of target concentrations of 10µM nitrate or ammonium and 1µM phosphate added to ambient seawater.

General apparatus of outdoor chemostat

Seaweed was maintained in on-growing apparatus (chemostat) for *Ulva* sp. as described in chapter 2.

Experimental design

I manipulated nutrient source (either ammonium or nitrate) and light availability (either shaded or ambient) in a fully crossed design. For experiment 1, I included three replicates for each treatment (a total of 12 separate chambers), and in experiment 2 and 3, I increased this to 4 replicates for each treatment. Positions of treatments within the chemostat were randomized.

'Shaded' treatment chambers were covered by 3 layers of 50% neutral density screen (a.k.a. mosquito mesh) giving an irradiance extinction coefficient of around 82 %. Light measurements were made underwater at the surface of the thalli using a Biospherical scalar irradiance (photosynthetic active radiation [PAR]) probe, model QSL2100.

Light levels at a weather station at the Leigh Marine laboratory were recorded over the duration of the experiments. Sea surface temperatures at the extraction point of the laboratory seawater flow through system were recorded daily.

Nutrient treatments consisted of either a 10µM nitrate and 1µM phosphate or 10µM ammonium and 1µM phosphate addition to seawater. Concentrations of ammonium (NH_4^+), nitrate (NO_3^-), and phosphate (PO_4^{3-}) in the seawater available in each experimental chamber were measured in triplicate in each chamber at the start of each 2 week experimental period. Sampling was repeated during the experimental periods to ensure the maintenance of seawater nutrient concentrations, except for experiment 3, where water samples were taken once at the conclusion of the trial. Samples were transferred to polycarbonate bottles and stored on ice until they could be analysed. Ammonium and phosphate concentrations were estimated following methods of Koroleff (1983), nitrate and nitrite were estimated using methods of Parsons et al. (1984).

Growth rates

To estimate growth rates, I weighed *Ulva* sp. specimens 3 times over 14 days during experiment 1, and 8 times over 14 days during experiments 2 and 3. Prior to

weighing, I removed excess moisture using a salad drier. After each weighing, tissue mass of all specimens was standardized to 3g by trimming. Algal growth rates in lab experiments were calculated for each time interval using a logistic growth equation:

$$W_t = W_0 \times e^{\mu t}$$

where W_t is the weight of algae at time t, W_0 the initial weight and μ the specific growth rate (d^{-1}).

Preparation and calculation of tissue %N

Whole plants were kept on ice until such time as they could be analysed, then cleaned of any epiphytes and epifauna, dried at 70˚C in a drying oven and ground to a fine powder. All isotope samples were analysed using a Europa Geo 20/20 isotope ratio mass-spectrometer interfaced to an ANCA-SL elemental analyser. Duplicate samples of 1.8g of powder were loaded into tin capsules for analysis of nitrogen content. The standard analytical error between duplicate analyses is lower than ±0.3‰ for nitrogen.

Preparation and calculation of tissue free amino acid content

Extraction of amino acids was performed following the methods of Barr and Rees (2003). I used perchloric acid for amino acid extraction, as this method immediately halts plant metabolism (Passonneau et al. 1979). I chopped all algal tissue finely prior to this addition, to homogenise the tissue sample and increase the surface area of the sample for more complete acid coverage. I conducted all of the extraction procedure on ice in order to minimise any enzyme activity not halted by the perchloric acid addition. I placed 1.0 g fresh weight (FW) of chopped alga in scintillation vials and added 5 ml 1 M perchloric acid, followed by 5ml (1 M KOH / 0.2 M MOPS) after 10 minutes to neutralise and buffer the solution. I then left the samples on ice for a

further 60 minutes to allow metabolites to leave the algal cells. I removed the algal tissue, and then centrifuged the remaining solution for 10 minutes at 1750 × g at 4 °C, decanted the supernatant and stored this at -80 °C before HPLC analysis. The external standards for this test were treated in the same manner as the samples. The amount of neutralising solution required to give pH 7 was determined by titration.

Amino acids were analysed following the methods of Barr and Rees (2003).
20 µl of amino acid extracts (above) were added to 10 µl lithium acetate. This solution was vortexed, centrifuged, frozen in liquid nitrogen and freeze-dried for 2 hours. The dry samples were then mixed with 30 µl of methanol:10% PITC (phenylisothiocyanate) in acetonitrile: triethylamine:water (7:1:1:1, v:v) and left at room temperature for 20 min. Following this, 150 µl of ultrapure water and 150 µl of heptane were added to each of the samples. The samples were stored at 4°C for less than 20 hours prior to HPLC analysis. Amino acids profiles were created using reversed phase chromatography on a Shimadzu high pressure binary HPLC system. Concentrations of amino acids were calculated using standard curves that were linear over the range of concentrations obtained from plant extracts.

Statistical analysis

I used two way analysis of variance (ANOVA) to test for differences in tissue %N values and tissue amino acid concentrations resulting from experimental manipulations of nutrients and/or light. Posterior pair wise comparisons between combinations of treatments were made using Tukey's HSD test to adjust for multiple comparisons. To test effects of treatments on growth rates of *Ulva* sp. over the duration of the experiments I used a series of two-way ANOVAs (to illustrate growth

trends at individual points during the experiments) and a repeated measures analysis (to best illustrate overall trend in growth over the duration of the trials). Significance estimates in repeated measures analysis are adjusted for repeat testing. All data satisfied the assumptions of normality and homogeneity of variances, and effect sizes were estimated where appropriate using r^2 values.

To test the hypothesis that growth rates of shaded plants receiving nitrate and ammonium sources converged over time (i.e. shaded plants suffered an initial cost to growth but 'acclimatised' to growth on nitrate), mean differences between these treatments during experiments 2 and 3 were tested for a trend using linear regression analyses.

Results

Nutrient levels

Levels of nitrate, ammonium and phosphate in algal chambers during experiments 1, 2 and 3 are shown in table 3.1. Shown are means and standard errors. n = 18 for each treatment in experiment 1, n = 24 for each treatment in experiment 2 and n = 16 for each treatment in experiment 3.

Table 3.1. Concentrations of nitrate, ammonium and phosphate in algal chambers. Mean values ± standard error

Concentration (µM)	Experiment 1				
		Treatment			
		Light		Shade	
	Ion	Nitrate / phosphate	Ammonium / phosphate	Nitrate / phosphate	Ammonium / phosphate
	NO_3^-	12.36 ± 0.16	0.14 ± 0.09	13.31 ± 0.16	0.12 ± 0.14
	NH_4^+	0.0 ± 0.05	12.85 ± 0.18	0.0 ± 0.03	12.20 ± 0.21
	PO_4^-	1.29 ± 0.07	1.38 ± 0.04	1.43 ± 0.05	1.26 ± 0.01
Concentration (µM)	Experiment 2				
		Treatment			
		Light		Shade	
	Ion	Nitrate / phosphate	Ammonium / phosphate	Nitrate / phosphate	Ammonium / phosphate
	NO_3^-	12.81 ± 0.67	2.08 ± 0.14	12.65 ± 0.49	2.06 ± 0.10
	NH_4^+	1.44 ± 0.05	12.27 ± 1.28	1.45 ± 0.07	12.56 ± 1.15
	PO_4^-	1.74 ± 0.03	1.84 ± 0.07	1.75 ± 0.07	1.92 ± 0.13
Concentration (µM)	Experiment 3				
		Treatment			
		Light		Shade	
	Ion	Nitrate / phosphate	Ammonium / phosphate	Nitrate / phosphate	Ammonium / phosphate
	NO_3^-	10.56 ± 0.23	0.1 ± 0.01	9.97 ± 0.22	0.1 ± 0.01
	NH_4^+	0.1 ± 0.1	10.54 ± 0.15	0.1 ± 0.1	9.45 ± 0.09
	PO_4^-	1.93 ± 0.01	1.94 ± 0.03	1.93 ± 0.00	2.02 ± 0.15

Experimental light and water temperature conditions

Daily solar radiation levels at the Leigh Marine Laboratory station during experiment 1 varied between 32.44 MJm^{-2} (day 2), and 19.46 MJm^{-2} (day 11). The mean daily radiation over the duration of the experiment was 27.48 MJm^{-2} ± 1.22 S.E (n = 14). Sea surface temperature ranged between 17.0 ºC (day 2) and 20.3 ºC (day 13). Mean sea surface temperature at the laboratory inflow point over the duration of the experiment was 18.7 ºC ± 0.25 S.E (n = 14). Daily solar radiation levels during experiment 2 varied between 33.02 MJm^{-2} (day 1), and 4.11 MJm^{-2} (day 15). The mean daily radiation over the duration of the experiment was 23.24 MJm^{-2} ± 2.19 S.E (n = 16). Sea surface temperature ranged between 18.4.0 ºC (day 1) and 20.2 ºC (day 11). Mean sea surface temperature at the laboratory inflow point over the duration of the experiment was 19.7 ºC ± 0.11 S.E (n = 16). Daily solar radiation levels during experiment 3 varied between 29.11 MJm^{-2} (day 7), and 7.07 MJm^{-2} (day 3). The mean daily radiation over the duration of the experiment was 21.37 MJm^{-2} ± 1.38 S.E (n = 15). Sea surface temperature ranged between 20.3 ºC (day 14) and 21.1 ºC (day 5). Mean sea surface temperature over the duration of the experiment was 20.6 ºC ± 0.06 S.E (n = 15).

Growth rates

Growth rates of *Ulva* sp. during experiment 1 were higher when algae were supplied with ammonium rather than nitrate during the first two of the three growth periods

(Tables 3.2, 3.3 and 3.4, Fig. 3.2). Growth during the third time period did not appear to differ between nitrate and ammonium fed algae. Growth rates of *Ulva* sp. during all periods of experiment 1 were significantly elevated in the presence of higher light. Post-hoc Tukey's HSD analysis showed that the mean growth rates of all light treatments were higher than for shade treatments, regardless of nutrient source. Overall, a repeated measures ANOVA indicated elevated growth rates of algae fed with ammonium versus nitrate (Table 3.5). Not surprisingly, algae in ambient light treatments grew significantly faster than shaded treatments over the period of the experiment. The significant differences in growth rates between nutrient treatments in the first two growth periods appear to be more pronounced in the shaded treatments (Fig. 3.2A). Repeated measures analysis of overall growth rates in experiment 2 (Fig. 3.3A and C) resolve significantly elevated growth rates in ambient light chambers versus shaded chambers, but fail to detect a significant effect of nitrogen source.

Table 3.2. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on growth rates in *Ulva* sp. (Experiment 1, Day 4)

Response factor	Source of variation	*df*	SS	MS	F	p
Growth rate	Nutrient	1	0.0035	0.0035	12.16	0.0082
	Light	1	0.0372	0.0372	130.39	<0.001
	Nutrient*light	1	0.00003	0.00003	0.0947	0.766
	Residuals	8	0.0023	0.0003		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	-0.0370	-0.0812	0.00715	0.1040
Ammonium/ Shade	Ammonium/ Light	-0.1143	-0.1585	-0.0702	<0.001
Nitrate/ Shade	Ammonium/ Light	-0.1453	-0.1895	-0.1012	<0.001
Ammonium/ Shade	Nitrate/ Light	-0.0773	-0.1215	-0.0332	0.0023
Nitrate/ Shade	Nitrate/ Light	-0.1083	-0.1525	-0.0642	<0.001
Nitrate/ Shade	Ammonium/ Shade	-0.0310	-0.0752	0.0132	0.1899

Table 3.3. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on growth rates in *Ulva* sp. (Experiment 1, Day 8)

Response factor	Source of variation	*df*	SS	MS	F	p
Growth rate	Nutrient	1	0.0029	0.0029	6.203	0.0375
	Light	1	0.0582	0.0582	125.32	<0.001
	Nutrient*light	1	0.0007	0.0007	1.518	0.2530
	Residuals	12	0.0037	0.0005		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	-0.0157	-0.0720	0.0407	0.8103
Ammonium/ Shade	Ammonium/ Light	-0.1240	-0.1804	-0.0676	<0.001
Nitrate/ Shade	Ammonium/ Light	-0.1703	-0.2267	-0.1140	<0.001
Ammonium/ Shade	Nitrate/ Light	-0.1083	-0.1647	-0.0520	0.0012
Nitrate/ Shade	Nitrate/ Light	-0.1547	-0.2110	-0.0983	<0.001
Nitrate/ Shade	Ammonium/ Shade	-0.0463	-0.1027	0.0100	0.1117

Table 3.4. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on growth rates in *Ulva* sp. (Experiment 1, Day 12)

Response factor	Source of variation	*df*	SS	MS	F	p
Growth rate	Nutrient	1	0.00006	0.00006	0.1111	0.7474
	Light	1	0.0575	0.0575	105.27	<0.001
	Nutrient*light	1	0.0004	0.0004	0.6441	0.4454
	Residuals	12	0.0044	0.0005		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	0.0063	-0.0548	0.0675	0.9865
Ammonium/ Shade	Ammonium/ Light	-0.1277	-0.1889	-0.0665	<0.001
Nitrate/ Shade	Ammonium/ Light	-0.1430	-0.2041	-0.0819	<0.001
Ammonium/ Shade	Nitrate/ Light	-0.1340	-0.1951	-0.0729	<0.001
Nitrate/ Shade	Nitrate/ Light	-0.1493	-0.2105	-0.0882	<0.001
Nitrate/ Shade	Ammonium/ Shade	-0.0153	-0.0765	0.0458	0.8512

Table 3.5. ANOVA analysis of growth rates - adjusted for repeated measures, experiment 1.

Source of variation	*df*	SS	F	p
Light	24	0.1514	350.4	<.0001
Nutrient	24	0.0048	11.181	0.0027
Time	24	0.0347	40.16	<.0001
Light x Nutrient	24	0.0005	1.242	0.276
Light x Time	24	0.0015	1.762	0.193
Nutrient x Time	24	0.0016	1.830	0.182
Light x Nutrient x Time	24	0.0005	0.634	0.539

Table 3.6. ANOVA analysis of growth rates - adjusted for repeated measures, experiment 2.

Source of variation	*df*	SS	F	p
Light	84	0.6260	462.8371	<.0001
Nutrient	84	0.0001	0.7212	0.3982
Time	84	0.0941	11.5961	<.0001
Light x Nutrient	84	0.0020	1.4543	0.2312
Light x Time	84	0.0373	4.6066	0.0004
Nutrient x Time	84	0.0088	1.0799	0.3811
Light x Nutrient x Time	84	0.0124	1.5292	0.1786

Figure 3.2

Figure 3.3

Repeated measures analysis of growth rates from experiment 3 (Table 3.7, Fig. 3.3 B and D) again show consistently higher growth rates in ambient light chambers, and no difference in growth rates between nutrient treatments.

Table 3.7. ANOVA analysis of growth rates - adjusted for repeated measures, experiment 3.

Source of variation	*df*	SS	F	p
Light	84	0.2842	174.07	<.0001
Nutrient	84	0.0004	0.2200	0.6403
Time	84	0.0990	10.110	<.0001
Light x Nutrient	84	0.0001	0.0346	0.8528
Light x Time	84	0.0458	4.6678	0.0004
Nutrient x Time	84	0.0097	0.9887	0.4383
Light x Nutrient x Time	84	0.0063	0.6460	0.6931

Regression analysis of the effect of nutrient source on growth rate through time (Fig. 3.4, Plot A) under shaded treatments in experiment 2 showed a steady reduction in the differences between treatments over time ($r^2 = 0.81$ for linear relationship). High variability in a comparative regression from experiment 3 (Fig. 3.4, plot B) reflect increases in within-treatment variability in this experiment.

Amino Acids

Levels of glutamine on day 9 of experiment 1 were significantly elevated when treated with ammonium relative to nitrate. Glutamine levels were also higher in ambient light treatments relative to shaded treatments. (Table 3.8, Fig. 3.5A). Post-

hoc paired comparisons using Tukeys HSD analysis showed concordant differences in glutamine levels between ammonium and nitrate-fed plants, regardless of light treatment (Table 3.8).

In contrast to patterns of glutamine on day 9, levels of glutamine levels in algae treated with ammonium or nitrate on day 14 of experiment 1 were indistinguishable (Table 3.9, Fig. 3.5B). Algae in ambient light were still enriched in glutamine relative to reduced light treatments.

Total Free Amino Acids (FAA) levels on day 9 of experiment 1 were significantly greater for ambient light and ammonium treated algae (Table 3.10, Fig. 3.6A). Post-hoc Tukey's HSD analysis gives evidence of significant differences in FAA content between ammonium and nitrate treatments in both light and shade.

ANOVA results of FAA levels on day 14 of experiment 1 (Table 3.11, Fig. 3.6B) in contrast, show no significant effects of light or nutrient treatment.

Figure 3.4

Figure 3.5

Figure 3.6

Table 3.8. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on glutamine content in *Ulva* sp. (Experiment 1, Day 9)

Response factor	Source of variation	*df*	SS	MS	F	p
Glutamine	Nutrient	1	5.880	5.8800	12.533	0.007
	Light	1	7.363	7.3633	15.695	0.004
	Nutrient*light	1	<0.0001	<0.0001	<0.001	1.00
	Residuals	8	3.753	0.469		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	-1.400	-3.190	0.390	0.134
Ammonium/ Shade	Ammonium/ Light	-1.567	-3.357	0.224	0.088
Nitrate/ Shade	Ammonium/ Light	-2.967	-4.757	-1.175	0.003
Ammonium/ Shade	Nitrate/ Light	-0.167	-1.957	1.624	0.990
Nitrate/ Shade	Nitrate/ Light	-1.567	-3.357	0.224	0.089
Nitrate/ Shade	Ammonium/ Shade	-1.400	-3.190	0.390	0.133

Table 3.9. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on glutamine content in *Ulva* sp. (Experiment 1, Day 14)

Response factor	Source of variation	*df*	SS	MS	F	p
Glutamine	Nutrient	1	<0.001	<0.001	<0.001	1.00
	Light	1	3.853	3.853	35.29	<0.001
	Nutrient*light	1	<0.001	<0.001	<0.001	1.00
	Residuals	8	0.873	0.109		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	<0.001	-0.863910	0.8639099	1.0000000
Ammonium/ Shade	Ammonium/ Light	-1.133	-1.997	-0.269	0.012
Nitrate/ Shade	Ammonium/ Light	-1.133	-1.997	-0.269	0.012
Ammonium/ Shade	Nitrate/ Light	-1.133	-1.997	-0.269	0.012
Nitrate/ Shade	Nitrate/ Light	-1.133	-1.997	-0.269	0.012
Nitrate/ Shade	Ammonium/ Shade	<0.001	-0.863910	0.8639099	1.0000000

Table 3.10. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on FAA content in *Ulva* sp. (Experiment 1, Day 9)

Response factor	Source of variation	*df*	SS	MS	F	p
FAA	Nutrient	1	5564.2	5564.2	41.87	<0.001
	Light	1	642.4	642.4	4.83	0.059
	Nutrient*light	1	8.0	8.0	0.060	0.812
	Residuals	8	1063.1	132.9		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	-44.70	-74.84	-14.55	0.006
Ammonium/ Shade	Ammonium/ Light	-16.27	-46.40	13.87	0.370
Nitrate/ Shade	Ammonium/ Light	-57.70	-87.84	-27.55	0.0013
Ammonium/ Shade	Nitrate/ Light	28.43	-1.708	58.57	0.064
Nitrate/ Shade	Nitrate/ Light	-13.00	-43.14	17.14	0.543
Nitrate/ Shade	Ammonium/ Shade	-41.43	-71.57	-11.29	0.001

Table 3.11. Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on FAA content in *Ulva* sp. (Experiment 1, Day 14)

Response factor	Source of variation	*df*	SS	MS	F	p
FAA	Nutrient	1	138.04	138.04	0.9953	0.347
	Light	1	428.41	428.41	3.0888	0.116
	Nutrient*light	1	480.07	480.07	3.4613	0.099
	Residuals	8	1109.57	138.70		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Nitrate/ Light	Ammonium/ Light	5.87	-24.93	36.66	0.926
Ammonium/ Shade	Ammonium/ Light	0.700	-30.09	31.49	0.999
Nitrate/ Shade	Ammonium/ Light	-18.67	-49.53	12.06	0.282
Ammonium/ Shade	Nitrate/ Light	-5.167	-35.96	25.63	0.947
Nitrate/ Shade	Nitrate/ Light	-24.60	-55.39	6.193	0.123
Nitrate/ Shade	Ammonium/ Shade	-19.43	-50.23	11.36	0.257

Figure 3.7

Tissue Nitrogen

There were no significant effects of nutrient and light treatment on algal nitrogen content after 9 days of treatment. At the conclusion of the experiment (14 days) ambient light treatments had significantly less tissue nitrogen relative to shaded treatments (Figure 3.7). Tukey's post-hoc comparisons between combinations of treatments gave no differences significant at a $p = 0.05$ threshold.

Discussion

Ecological Implications

The initially slower response of algal growth rates and nitrogen assimilation of nitrate-fed algae suggests that growth rates of natural populations of *Ulva* sp. may respond more slowly to episodic pulses of nitrate than to ammonium. However, the temporary nature of this effect indicates that growth rates in steady-state populations receiving different proportions of their nitrogen supply via continuous enrichment from nitrate and/or ammonium sources are likely to be similar.

Impact of nutrient source on growth rates of *Ulva* sp. under light and dark conditions

Overall, the impact of nitrate reduction on nitrogen assimilation and growth in *Ulva* sp. appeared to be temporary. Perhaps due to the level of natural variability in growth rates associated with this system, there was no evidence to suggest an interaction between light level and nutrient type on growth rates.

The observation that growth rates appeared indistinguishable as a function of nutrient treatments at any stage of experiment 3 suggests that any long-term growth cost resulting from nitrate reduction is obscured by natural variability in growth rates within this system.

Some works have suggested a theoretical advantage to growth on ammonium rather than nitrate. Raven et al. (1992) and Raven (1984, 1987a) suggest a greater cost in photons per unit carbon assimilated during growth on nitrate than would be the case for ammonium, and the need for more of the product of this reduction (ammonium) to create catalysts required for this reaction in new cells. If these costs affected growth rate in this case, the first would be specific to light limited algae, and the second would impact on growth rates at any light level. My findings, however, suggest little lasting effect of nitrate reduction on growth rates in *Ulva* sp. The absence of a significant statistical interaction between nutrient and light on growth rates at any period during these experiments suggests that a growth-per-photon cost of nitrate reduction is minor, at least relative to other sources of variation. However a significant effect of nutrient type on the overall growth rate at the beginning of experiment 1 and 2 suggests the sum of these costs is (at least temporarily) sufficient to slow growth. A third possibility may be that in the long term, both of these costs are minor, but the growth reduction I observed in these experiments reflects the energetic cost of creating enough catalyst in all tissue present at the start of the experiment to facilitate growth on a 'nitrate-only' N supply.

The apparent increase in the within-treatment variance in all light chambers, and in all growth rates in experiment 3 may be attributable to UV impacts on the health of the

experimental algae, a result of shifting a typically sub-tidal alga to a high UV environment (Vinegla et al. 2006).

Impact of light and dark conditions on free amino acid (FAA) content of *Ulva* sp.

It has previously been shown that under low light conditions, the reduction of nitrate to ammonium can become a 'rate-limiting' step in the assimilation of nitrogen by algae (Wada and Hattori 1978, Needoba et al. 2004). Where this occurs, algae may effuse unreduced nitrate (Wada and Hattori 1978, Needoba et al. 2004), or store it in an unreduced form (Raven 1987a). However, in situations of excess nitrogen supply where sufficient light is present, algae may reduce the nitrate and store it in a variety of forms, including amino acids, chlorophyll, and protein. Differences in the presence of amino acids in the tissue of nitrate and ammonium-fed *Ulva* sp. in these experiments are taken to represent the ability of these algae to reduce available nitrate. Tissue glutamine levels taken on day 9 of experiment 1 showed a significant effect of nitrogen source, primarily as a result of differences between shaded plants. This significant effect had attenuated by day 14. Glutamine, as the primary amino acid in the nitrogen assimilation chain (Vergara et al. 1995) in this case acts as a first indicator of the process of nitrate reduction. I suggest that the lower values of glutamine on day 9 reflect the greater energetic cost associated with nitrate assimilation, and the subsequent reduction in the differences between these values reflects acclimatisation to an all-nitrate nitrogen source.

The patterns evident in the total free amino acid pool of *Ulva* sp. tissue show similar patterns to those evident in glutamine pools, however it appears that the shaded nitrate

treatment may be slightly slower to recover to the levels of other treatments than was the case with glutamine (compare Fig. 3.5 A, B with Fig. 3.6 A and B). A weak interaction effect in the response of FAA to light level and nitrogen source (Table 3.11) may indicate that in this case differences in incident photons may be impacting the ability of *Ulva* sp. to fully reduce excess nitrate to amino acids as recorded in some seaweeds in natural populations existing in low light conditions (Raven et al. 1992). In this case a lack of energy may hinder the reduction of nitrate to nitrite and ammonium as shown in Figure 3.1.

Impact of nutrient source on tissue nitrogen

Algae in this experiment were supplied with excess nitrogen and phosphorus, in order to highlight light limitation and the cost of reducing nitrate to ammonium as a nitrogen source. Lower tissue nitrogen content of high light plants reflects increased growth rates under high light, relative to rates of nitrogen uptake. However tissue nitrogen in both high and low light treated algae, (reflected in the percentage of nitrogen in the total dry weight of tissue,) were high enough that nitrogen limitation was unlikely to have any effect on growth rates at any time during the experiment for any treatment (compare Fig. 3.7 A, B with Fig. 2.14).

A previous study on the nitrogen content response of *Ulva fenestrata* to increases in nitrate and ammonium found a greater increase in tissue %N during growth on ammonium than on nitrate (Naldi and Wheeler 1999). Naldi and Wheeler's study also measured tissue nitrogen content after 9 days of growth, and generated tissue nitrogen concentrations of 4.2% and 4.7% for nitrate and ammonium, respectively. Algae in

this study were also grown under conditions of relatively low light availability (around 100μEm^{-2}s^{-1}), and showed lower FAA pools in nitrate than in ammonium treatments. The nitrate treatment also generated significant intracellular pools of unreduced nitrate. This may suggest that nitrogen assimilation in this study was also reduced for nitrate relative to ammonium due to reduced nitrate reduction capacity in low light conditions.

Conclusions and further research

Overall there was a temporary reduction of the growth rate and nitrogen assimilation capacity of *Ulva* sp., and I suggest this is indicative of the added cost of reducing nitrate to ammonium. This negative effect on growth was not present after around 10 days of growth in ambient light or shaded conditions, to the point where growth rates on nitrate and ammonium were statistically indistinguishable. This initial impact on growth rate was mirrored by an initial lag in the production of glutamine, a primary amino acid in the nitrogen assimilation chain, and FAA content.

This information suggests that the cost in photons of nitrate reduction is unlikely to have lasting impacts on the growth of *Ulva* sp. However, these data do not give information about the exact nature of the initial growth reduction. Possibilities for further research exist in investigating this effect using a time series of amino acid profiles, examining particularly the presence of malate, one of the primary energy transfer molecules in algae (Scheibe 2004), to further confirm that this reduction in growth rate is due to energy limitation. Also, to better understand the nature of this initial growth reduction, the nature of changes in nitrate and ammonium uptake during

this time need to be examined. It is possible that the initial growth reduction in nitrate-fed plants reflects a standard cost of reduction per mol nitrate, but is increased due to an initial rapid uptake of nitrate.

Chapter 4 – $\delta^{15}N$ and $\delta^{13}C$ ratios as tracers of the dissolved and particulate fractions of sewage effluent

Abstract

Particulate and dissolved fractions of sewage effluent may disperse differently, be assimilated by different organisms, and have different impacts on marine ecosystems. Patterns of dissolved and particulate effluent dispersal can be estimated separately using $\delta^{15}N$ and $\delta^{13}C$ ratios in species that assimilate sewage dissolved inorganic nitrogen (DIN) (a marine alga, *Carpopyllum maschalocarpum* and its epifaunal grazer, *Amphiroidea media*), and sewage particulate organic matter (POM) (a water column filter-feeder, *Petrolisthes elongatus*) over a gradient of effluent influence away from a point source discharge. Sewage concentration was estimated by the presence of dissolved nitrogen and phosphate ions, and particulate matter in seawater. Tissue $\delta^{15}N$ levels measured for the alga, grazer and filter feeder all increased significantly with increasing sewage influence as estimated from dissolved ion concentrations, from (means ± standard error) 5.63‰ ± 0.41, 7.36‰ ± 0.33 and 9.16‰ ± 0.11 at the site with the lowest sewage influence to 13.35‰ ± 0.39, 10.81‰ ± 0.28 and 11.97‰ ± 0.16 at the site with the highest sewage influence, reflecting assimilation of ^{15}N-enriched sewage DIN (where $\delta^{15}N$ ratio of sewage DIN was 23.43 ± 2.14‰). Filter feeder $\delta^{13}C$ ratios generally became more depleted with increasing seawater turbidity, and ranged from -22.6‰ ± 0.19 to -17.48‰ ± 0.74, reflecting the dispersal and assimilation of ^{13}C-depleted sewage particulates (where $\delta^{13}C$ sewage POM was -27.4‰ ± 0.76).

Introduction

Expansions of coastal communities worldwide have caused increases in pressures responsible for environmental change in marine environments (GESAMP 2001). Increases in sewage influence can impact on marine biota directly (e.g., through the influence of organochlorides and heavy metals (Addison 1976, Bryan 1976), and indirectly (e.g., through ecosystem changes resulting from nutrient enrichment (Bokn et al. 2002, Hauxwell et al. 2003, Karez et al. 2004, Tewfik et al. 2005). However, sewage impacts on species diversity and abundance can be obscured statistically by natural variability in marine community structure (Ellis et al. 2000, Hewitt et al. 2005). It is therefore important to develop indirect measurements of sewage impact.

Sewage, and other terrestrially derived matter, tends to contain carbon and nitrogen that is isotopically distinct from that found in unimpacted coastal marine ecosystems (Gartner et al. 2002, Bedard-Haughn et al. 2003, Gordon and Goni 2003, Savage and Elmgren 2004). Stable isotope ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$ ratios) in marine biota can provide a method of tracing patterns of terrestrial nutrient sources entering marine ecosystems, as the $\delta^{13}C$ and $\delta^{15}N$ ratios of nutrient sources tend to be transferred predictably to the organisms that assimilate them (Fry and Sherr 1984, Fry and Wainright 1991).

Stable isotope levels in the tissues of all marine organisms that assimilate sewage-derived nutrients are valuable as measures of sewage impact because they act as time-integrative measures of sewage presence, reflecting the contributions of available nutrient sources over the period of tissue generation and turnover (Gartner et al. 2002,

Gaston and Suthers 2004, Perga and Gerdeaux 2005). In contrast, measurements of sewage presence made directly from the water column provide an instantaneous measure of sewage dispersal. Rare pulses of sewage effluent missed by water column sampling may still be ecologically significant (Wallentinus 1984, Valiela et al. 1997).

The value of stable isotope signatures as tools in sewage impact assessment relies on knowledge of how the signatures in study organisms respond to patterns of sewage concentration. $\delta^{13}C$ and $\delta^{15}N$ signatures in consumer tissues can then be used as a quantitative measure to link ecological effects in impacted areas to the magnitude of sewage influence. The particulate fraction of sewage effluent has the potential to impact on marine systems differently to dissolved effluent (Bryan 1976), and dispersal of particulates cannot be assumed to co-vary with concentrations of dissolved sewage components (Ellis 1989).

Previous research has suggested that marine macroalgae are particularly suited to detect the presence of dissolved inorganic nitrogen (DIN) from sewage (McClelland and Valiela 1998, Gartner et al. 2002), and that filter-feeding organisms affected by sewage will assimilate the distinct $\delta^{13}C$ and $\delta^{15}N$ signatures of sewage particulate organic matter (SPOM) (Rogers 2003, Dolenec et al. 2006, Piola et al. 2006).

Preferential assimilation of one isotope over the other during assimilation may result in differences in isotopic signatures between the organism assimilating the nutrients, and the $\delta^{13}C$ and $\delta^{15}N$ signatures of the nutrient pools (fractionation). For consumers, fractionation in carbon and nitrogen isotopes is commonly used to estimate trophic level, based on standard estimates of fractionation as tissue is broken down and

assimilated at each step in the trophic chain. An increase in trophic level normally results in an increase in $\delta^{15}N$ ratio of the consumer over its diet of between 2.2 and 3.4‰ (Vander Zanden and Rasmussen 2001, McCutchan et al. 2003). For carbon, fractionation is typically below 1‰ (DeNiro and Epstein 1976). However, fractionation in consumers has been observed to vary between species, and functional groups (Gannes et al. 1997, Vanderklift and Ponsard 2003, Fry 2006). Differences in fractionation between animals may differ due in part to differences in excretion rate (Mill et al. 2007). For algae, fractionation differences have been observed to differ between species, and as a result of the physical conditions in which the algae grow (Needoba and Harrison 2004, Cornelisen et al. 2007).

The aim of this work is to describe patterns of particulate and dissolved fractions of sewage effluent dispersal separately using $\delta^{13}C$ and $\delta^{15}N$ ratios in marine organisms that assimilate their carbon and nitrogen from either the particulate fraction (a filter feeder of water column POM (*Petrolisthes elongatus*) or the dissolved fraction (a macroalga (*Carpopyllum maschalocarpum*) and its epifaunal herbivore (*Amphiroidea media*).

I hypothesise that the filter feeder (*Petrolisthes elongatus*) will display contrasting patterns of $\delta^{13}C$ and $\delta^{15}N$ signatures to those of the macroalga (*Carpopyllum maschalocarpum*) and the algal grazer (*Amphiroidea media*), and that these patterns will reflect the movement and assimilation of particulate sewage $\delta^{13}C$ and $\delta^{15}N$ signatures into the filter feeder, and sewage DIN $\delta^{15}N$ signatures into the macroalga and grazer. These measurements are made over a measured concentration gradient of

dissolved and particulate sewage influence surrounding the Titahi Bay wastewater treatment plant (TBWWTP) outflow, near Wellington, New Zealand.

Methods

Quantifying environmental variation

I measured water turbidity at all sites (n=11, see Fig 2.1) on 5 occasions during December, January and February 2005 to approximate the dispersal patterns of effluent particulates around the TBWWTP. The TBWWTP releases effluent with a concentration of between 3 and 30mg/L of suspended solids, and is the only major inflow of terrestrial waters in the vicinity of the sampling area.

Measurements were made using an RBR XR420 turbidity meter. Measurements were made in surface waters (i.e., 0.5 m depth) and at 4 m depth. The turbidity meter was programmed to record at 5 second intervals and was deployed in surface water and subsequently at 4 m for a period of 1 minute in each location. Turbidity estimates were generated by time-averaging data acquisitions over the period of deployment (1 minute = 12 data acquisitions). To separate turbidity from the wastewater outflow point source from natural between-day variation in sea turbidity, averages at each site and date were standardised to z values based on the daily mean and standard deviation of turbidity at all sites.

Measurements and characterisation of dispersal patterns of the dissolved components of the TBWWTP sewage plume are covered in chapter 2.

Sampling primary producers and consumers and their stable isotope signatures

I sampled a primary producer (a macroalga, *Carpophyllum maschalocarpum;* 3 samples per site) and two primary consumers (grazer: *Amphiroidea media*; 3 samples per site; filter feeder: half crab, *Petrolisthes elongatus*; 3 samples per site) in a distance gradient away from the TBWWTP discharge (Fig. 2.1) in March, April and May 2005.

Algae were collected from a depth of ~2 m, within 20 m from shore (immediately inshore from distance gradient sites marked by buoys). All algal samples were maintained on ice in the field, except during epifauna removal, and subsequently frozen until they could be analysed. Samples of *A. media* were collected from the algal samples used in isotope analysis for each site and date. To remove all animals (including *A. media*) from the algal samples, algae were left in plastic bags in the sun for 30 minutes until their epifaunal communities died of heat stress. I then rinsed the animals from the algae in buckets of freshwater, filtered the water through 156 µm plankton mesh, selected out the individuals of *A. media* from the mesh and froze them until they could be analysed further. *Petrolisthes elongatus* samples were collected from beneath rocks around the low tide mark, within 20 m of algal collection points.

Preparation of tissue samples for isotope and percent carbon and nitrogen analysis

To calculate estimates of nitrogen isotope signatures within *C. maschalocarpum*, frozen samples were cleaned of any epiphytes, dried at 70°C in a drying oven and ground to a fine powder. Tissue was collected from a homogenized sample of one entire macroalgal specimen, minus thallus and holdfast, to provide a standard

relationship between consumer and producer isotope ratios. Previously frozen *A. media* and *P. elongatus* samples were rinsed in freshwater, dried in a drying oven and ground to a fine powder. In order to generate sufficient tissue for isotope analysis of each sample, 10 individuals of *A. media* and *P. elongatus* were homogenized to create single composite samples for each species, site and date. All 10 *A. media* individuals from each site and date were removed from the single specimens of *C. maschalocarpum* used in isotope analysis.

Carbon incorporated into tissue and inorganic carbonate are of different origin and therefore different quantities of inorganic carbon in samples would bias analysis of results (Fry 1988, Cloern et. al. 2002). Therefore it was necessary to remove all inorganic carbon by acidification. Dried and ground algal and epifaunal samples were acidified with 1 mol l^{-1} hydrochloric acid (HCl) using the drop by drop method according to Jacob et al. (2005), re-dried at 70°C and re-ground prior to spectrophotometry.

All isotope samples were analysed using a Europa Geo 20/20 isotope ratio mass-spectrometer interfaced to an ANCA-SL elemental analyser. Duplicate samples of 1.8mg of powder were loaded into tin capsules for analysis of organic carbon and nitrogen content and carbon and nitrogen isotopic composition. The standard analytical error between duplicate analyses is lower than ±0.3‰ for nitrogen and ±0.1‰ for carbon. Relative nitrogen isotopic concentrations are reported as $\delta^{15}N$ values relative to an air standard, where

$$\delta^{15}N‰ = \left(\frac{{}^{15}N/{}^{14}N_{sample} - {}^{15}N/{}^{14}N_{air}}{{}^{15}N/{}^{14}N_{air}} \right) \times 1000$$

Relative carbon isotopic values are reported as $\delta^{13}C$ ratios relative to a VPDB standard,

$$\delta^{13}C‰ = \left(\frac{{}^{13}C/{}^{12}C_{sample} - {}^{13}C/{}^{12}C_{VPDB}}{{}^{13}C/{}^{12}C_{VPDB}} \right) \times 1000$$

where VPDB is an international $^{13}C/^{12}C$ standard of cretaceous belemnite from the Peedee formation in South Carolina, USA. Percent carbon and nitrogen values for algal tissue are given for dry weights.

Collection and preparation of samples for source signature ^{15}N and ^{13}C analysis

To characterise the isotopic composition of effluent discharged from the TBWWTP, I sampled primary and tertiary effluent at weekly intervals (n=3) in March 2005. Sample processing and $\delta^{15}N$ estimates for dissolved nitrogen were acquired as detailed in chapter 2. $\delta^{13}C$ values of dissolved inorganic carbon (DIC) were not included in this study as a possible tracer of dissolved sewage effluent dispersal. High concentrations of enriching nutrients (N and P) in effluent have the potential to impact the growth rates and ^{13}C assimilation tendencies of the primary producers which might be used to track the movement of effluent DIC into marine food chains. For analysis of effluent particulate $\delta^{15}N$ and $\delta^{13}C$ ratios, effluent samples were filtered onto Whatman 0.7 μm GFF glass fibre filters and the particulate portion was dried at 70°C before carbon and nitrogen isotope analysis.

Statistical analysis

I examined spatial patterns of turbidity around the TBWWTP using ANCOVA analysis. I employed distance from the outflow as a covariate in this analysis, and direction from the outflow as a factor at two levels.

Previous dietary research suggests that *A. media* is herbivorous, and *C. maschalocarpum* is likely to form a significant part of its diet (Robbins 1990). In order to test that this dietary tendency resulted in *A. media* following spatial patterns of $\delta^{15}N$ and $\delta^{13}C$ in its algal food source (and hence tracking increases in the availability of sewage DIN) I examined co-variance in $\delta^{15}N$ and $\delta^{13}C$ patterns in samples of the two species formally using linear regression. *A. media* isotope ratios were fitted against those of the *C. maschalocarpum* algae they were collected from.

Because my previous analyses suggested that sewage concentrations decrease with increasing distances from the TBWWTP outflow, and for dissolved constituents were biased toward the westward direction, I explored spatial variation in tissue $\delta^{13}C$ and $\delta^{15}N$ within each study organism using ANCOVA models where: (1) distance from the outfall was included as a covariate, and (2) direction (east or west) from the outflow was included as a categorical variable. The full ANCOVA models included an interaction term. Data were checked for normality and homogeneity of variance, and where appropriate dependent variables were log transformed to improve homogeneity of variances.

Results

$\delta^{15}N$ and $\delta^{13}C$ signatures of particulate and dissolved constituents of sewage effluent

Overall, tertiary processed (final) effluent at the TBWWTP showed enriched DIN $\delta^{15}N$ (23.4‰ ± 2.1 S.E.) and comparatively depleted particulate $\delta^{15}N$ signatures (6.8‰ ± 1.2 S.E.) (Fig. 4.1A). Particulate $\delta^{13}C$ signatures in final effluent had low $\delta^{13}C$ ratios (-27.4‰ ± 0.76 S.E.), and particulate $\delta^{13}C$ did not appear to be greatly affected by tertiary processing (Fig. 4.1B). $\delta^{15}N$ levels of primary processed dissolved (7.3‰ ± 1.6 S.E.), and particulate (5.1‰ ± 1.8 S.E.) effluent fractions were more depleted in ^{15}N than for tertiary processed effluent. For more detail on tertiary processing effects and between-treatment plant variability in effluent $\delta^{15}N$ and $\delta^{13}C$ signatures see appendix 5.

Spatial variation in dissolved and particulate sewage effluent components

Dissolved effluent components tended to be more concentrated in water sampled in a westerly direction, and at shorter distances from the TBWWTP outflow. These trends in dispersal of dissolved components of TBWWTP sewage effluent are covered in more detail in graphical and MANOVA analyses presented in chapter 2 (Fig. 2.4, Table 2.1). Graphical representation of turbidity measurements suggest a similar westward, surface concentrated pattern of dispersal of sewage particulate matter to that of dissolved effluent components (Fig. 4.2). ANCOVA analysis of trends in spatial variation in turbidity around the TBWWTP were limited by high between-day variability in turbidity (i.e. high residual SS), however there was a

Figure 4.1

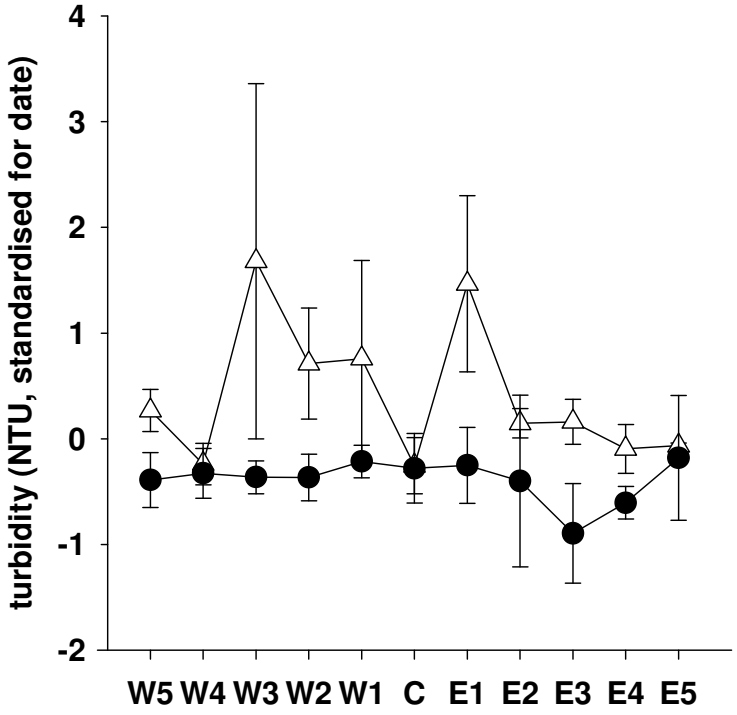

Figure 4.2. Turbidity in surface waters (white triangles) and at 4m depth (black circles) Site 'C' is at the WWTP outflow, sites marked W1-W5 are at increasing distances to the west of the outflow. Sites marked E1-E5 are at increasing distances to the east of the outflow. Error bars +/- S.E. n = 5.

weak effect of increasing distance from the TBWWTP outflow on reducing turbidity (Table 4.1).

Table 4.1. ANCOVA analysis of water column turbidity change with distance, and direction from the TBWWTP.

Response factor	Source of variation	*df*	SS	MS	F	p
Turbidity	Direction	1	1.645	1.645	1.647	0.2073
	Distance	1	2.79	2.79	2.7942	0.103
	Residuals	37	36.945	0.999		

Variation in $\delta^{13}C$ signatures recorded in a primary producer, algal grazer and a water column filter feeder

Overall $\delta^{13}C$ values of *C. maschalocarpum* were not significantly different as a result of direction (east or west from the outflow), or with increasing distance from the outflow pipe. However, there was a significant interaction term in this ANCOVA analysis, resulting from a tendency toward lower $\delta^{13}C$ values at western sites nearest the outflow, as well as the most distant sites to the east of the outflow (Table 4.2, Fig. 4.3 and 4.4). Overall $\delta^{13}C$ values of *A. media* were also not significantly different as a result of direction (east or west from the outflow), or with increasing distance from the outflow pipe. There was also a significant interaction term in this ANCOVA analysis, as *A. media* followed a similar trend to *C. maschalocarpum*, showing depleted $\delta^{13}C$ values at western sites nearest the outflow and most distant sites to the east of the outflow (Table 4.2, Fig. 4.3 and 4.4). $\delta^{13}C$ values of organic *A. media* tissue showed a significant, but loose relationship with those of the *C. maschalocarpum* plants that they were collected from (Fig. 4.5, plot B).

Table 4.2. ANCOVA analysis of tissue $\delta^{13}C$ signature change with distance, and direction (east or west) from the TBWWTP.

Response factor	Source of variation	*df*	SS	MS	F	p
$\delta^{13}C$ *C. maschalocarpum*	Direction	1	6.787	3.393	3.252	0.054
	Distance	1	0.036	0.036	0.035	0.854
	Distance* Direction	1	36.08	36.08	34.57	<0.001
	Residuals	26	29.223	1.044		
$\delta^{13}C$ *A. media*	Direction	1	0.1153	0.0576	0.0833	0.920
	Distance	1	1.007	1.007	1.455	0.238
	Distance* Direction	1	7.091	7.091	10.24	0.0034
	Residuals	26	19.38	0.692		
$\delta^{13}C$ *P. elongatus*	Direction	1	31.97	15.99	18.35	<0.001
	Distance	1	24.99	24.99	28.69	<0.001
	Distance* Direction	1	2.993	2.993	3.4352	0.0744
	Residuals	26	24.39	0.871		

Table 4.3a Linear regression analysis of $\delta^{15}N$ ratios in individual *C. maschalocarpum* algae, and $\delta^{15}N$ ratios in tissue of *A. media* removed from them.

	Coefficient	Std error	T	p
Intercept	4.0210	0.6934	5.7985	<0.0001
Slope	0.5768	0.0716	8.0569	<0.0001

Correlation coefficient				
Source	df	MS	F	p
Regression	1	53.7188	64.9138	<0.0001
Residual	32	2.4804		

Table 4.3b Linear regression analysis of $\delta^{13}C$ ratios in individual *C. maschalocarpum* algae, and $\delta^{13}C$ ratios in tissue of *A. media* removed from them.

	Coefficient	Std error	T	p
Intercept	-10.2476	1.2837	-7.9828	<0.0001
Slope	0.2410	0.1023	2.3558	0.0250

Correlation coefficient				
Source	df	MS	F	p
Regression	1	4.1899	5.5498	0.0250
Residual	32	0.8623		

Figure 4.3

Figure 4.4

Figure 4.5

P. elongatus was highly depleted in ^{13}C in high sewage impact areas to the west and at shorter distances from the outflow (Table 4.2, Fig. 4.6 and 4.7). $\delta^{13}C$ values in *P. elongatus* showed a larger range than *A. media* or *C. maschalocarpum,* and appeared to follow the general pattern of effluent dispersal evidenced by dissolved effluent components and seawater turbidity.

Variation in $\delta^{15}N$ signatures recorded in a primary producer, algal grazer and a water column filter feeder

$\delta^{15}N$ values of *C. maschalocarpum* tissue showed patterns of enriched $\delta^{15}N$ signatures to the west, and at sites close to the outflow (Table 4.4, Fig. 4.6 and Fig. 4.7). *C. maschalocarpum* $\delta^{15}N$ signatures ranged between 5.63‰ ± 0.41 S.E. at the most ^{15}N-depleted site (Site E5) and 13.35‰ ± 0.39 S.E at the most ^{15}N-enriched site (Site W2). *A. media* tissue also showed enriched $\delta^{15}N$ signatures to the west and at sites closer to the outflow (Table 4.4, Fig. 4.6, Fig. 4.7, and Fig. 4.8). Additionally, $\delta^{15}N$ signatures of *A. media* showed a consistent relationship with those of with *C. maschalocarpum*, which was linear over the range of values recorded (Fig. 4.5, Plot A). Filter feeder *(P. elongatus)* tissue also showed significantly enriched $\delta^{15}N$ signatures in the high sewage impacted areas to the west and at short distances from the outflow (Table 4.4, Fig. 4.6 and 4.7). These increases were also strongly correlated with $\delta^{15}N$ enrichment in the primary producer *C. maschalocarpum*, (i.e. with patterns of sewage DIN dispersal) (Fig. 4.5).

Figure 4.6

Figure 4.7

Figure 4.8

Table 4.4. ANCOVA analysis of tissue $\delta^{15}N$ signature change with distance, and direction (east or west) from the TBWWTP.

Response factor	Source of variation	*df*	SS	MS	F	p
$\delta^{15}N$ *C. maschalocarpum*	Direction	1	110.4	55.18	46.60	<0.001
	Distance	1	13.76	13.76	11.62	0.0020
	Distance* Direction	1	4.194	4.194	3.541	0.0703
	Residuals	26	33.86	1.167		
$\delta^{15}N$ *A. media*	Direction	1	57.25	28.63	46.68	<0.001
	Distance	1	2.287	2.287	3.730	0.064
	Distance* Direction	1	2.664	2.664	4.344	0.046
	Residuals	26	17.170	0.613		
$\log\delta^{15}N$ *P. elongatus*	Direction	1	0.2419	0.1209	121.8	<0.001
	Distance	1	0.0294	0.0294	29.64	<0.001
	Distance* Direction	1	0.0006	0.0006	0.5722	0.456
	Residuals	26	0.0621	0.0021		

Discussion

Between-species differences in tissue $\delta^{13}C$ and $\delta^{15}N$ response to sewage effluent

C. maschalocarpum showed patterns of elevated $\delta^{15}N$ signatures that correspond to areas of predicted high effluent concentration. No measures of 'clean' oceanic DIN $\delta^{15}N$ signatures were made during this study due to the impractically large quantity of water required to get a signature measurement from oligotrophic waters (Gartner et al. 2002). However previous studies performed in temperate coastal waters suggest that background oceanic and coastal DIN signatures range between ~ 6 and 7‰, (Miyaki and Wada 1967, Sigman et al. 1997). $\delta^{15}N$ levels in *C. maschalocarpum* taken from clean environments (Mokahinau Islands, Leigh and Poor Knights Islands) in New Zealand ranged between 5.3 and 7.6‰ (pers. com Neill Barr).

It is likely that *C. maschalocarpum* tissue $\delta^{15}N$ signatures from sites 300-500 metres to the east of the outflow (5.6 - 7.1‰) represent relatively unimpacted conditions. Macroalgae are generally reliant on water column DIN to meet their nitrogen requirements (Wallentinus 1984), and the increase in *C. maschalocarpum* $\delta^{15}N$ signatures at short distances from the outflow suggests that sewage DIN (23.4‰ ± 2.1 S.E.) forms an increasing portion of their nitrogen budget at these sites.

C. *maschalocarpum* tissues showed the largest range in $\delta^{15}N$ signatures of the three species tested, indicating sensitivity to the presence of ^{15}N-enriched sewage effluent.

Depleted $\delta^{13}C$ values in *C. maschalocarpum* at some sites are potentially the result of increases in the availability of effluent DIC. DIC concentrations in sewage effluent are generally higher than those in seawater (Llorens et al. 1993, Ballester et al. 1999),

and the dilution of DIN concentrations from tertiary processed effluent sewage to seawater at sampling sites W2 and E1 indicate that sewage was typically diluted by only ~90% within 50-100 metres of the TBWWTP outflow (compare Fig. 2.2 with 2.4). However, sewage DIC assimilation to algal tissue does not offer the same potential as a tracer of sewage dispersal as the assimilation of sewage DIN. Because nitrogen is generally present in very low concentrations in clean seawater in the Titahi Bay area relative to their concentrations in sewage (tertiary processed sewage leaving TTBWWTP has around 200 times the TIN concentration of seawater at low impact sites in this study), nitrogen from effluent can contribute a large proportion of the nitrogen available to algae despite dilution. While CO_2 is slightly more soluble in freshwater than saltwater, assuming that sewage derived $CO_{2(aq)}/HCO_3^-$ follow similar patterns of dilution to nitrogen and phosphate ions, it is likely that $CO_{2(aq)}/HCO_3^-$ from sewage effluent contributed only a small proportion of DIC available to *C. maschalocarpum* at high-impact sites. Notably, the ^{13}C-depletion of *C. maschalocarpum* at high-impact sites is in contrast to the previously recorded ^{13}C-enrichment in *Ulva* sp. at high impact sites and in high nitrogen laboratory trials in chapter 2, and may suggest that *C. maschalocarpum* has less physiological demand for CO_2 (potentially slower growth rates) at high impact sites. Additionally, *C. maschalocarpum* at some low impact sites also showed depleted $\delta^{13}C$ values (Fig. 4.3).

A. media showed spatial patterns of elevated $\delta^{15}N$ signatures that correspond to areas of high predicted effluent concentration. Spatial patterns of *A. media* $\delta^{15}N$ and $\delta^{13}C$ were correlated with patterns shown in its host alga, *C. maschalocarpum.* $\delta^{13}C$ ratios of *A. media* were, however, comparatively stable over the spatial scale examined here,

and $\delta^{15}N$ showed a lower range of values than *C. maschalocarpum* in response to increasing predicted effluent influence. Where a direct trophic link between the alga and its herbivorous epifauna would typically result in $\delta^{13}C$ and $\delta^{15}N$ increases (enrichment) of ~ 1 ‰ and 2.2 – 3.4 ‰ for carbon and nitrogen, respectively (DeNiro and Epstein 1976, Vander Zanden and Rasmussen 2001, McCutchan et al. 2003), this is not evident in the isotope ratios of *C. maschalocarpum* and *A. media* (Fig. 4.3 and 4.4). $\delta^{13}C$ ratios of *A. media* were on average 0.79‰ lower than those of *C. maschalocarpum.* At relatively unimpacted sites to the east of the outflow point, *A. media* show $\delta^{15}N$ ratios around 1-2‰ above those of their host plant. This relationship is obscured in sites receiving higher sewage concentration to the west of the outflow. While the patterns of isotope ratios in Figures 4.3 and 4.4 do not expressly distinguish *C. maschalocarpum* as a dietary component of *A. media*, they do indicate a degree of shared isotopic response of the two species to patterns of sewage influence. As the sampling procedure used to generate $\delta^{15}N$ and $\delta^{13}C$ values representative of *C. maschalocarpum* tissue used a homogenised sample of the entire alga, minus thallus and holdfast, it is possible that the diet of *A. media* was separated from this value based on selective feeding on certain parts of the alga. $\delta^{13}C$ values of brown algae have been noted to vary across tissue types (Stevenson et al. 1984). Alternatively, the shared patterns of $\delta^{15}N$ and $\delta^{13}C$ values in *A. media* and *C. maschalocarpum* may indicate *C. maschalocarpum* as a partial component of the diet of *A. media,* or that other algal species (forming some part of its diet) in the vicinity of the host alga shared similar physical and chemical conditions for growth, and so shared patterns in isotopic composition.

P. elongatus showed increased $\delta^{15}N$ (range ~3‰) and strongly decreased $\delta^{13}C$ (range ~6‰) in areas of high effluent influence. Because the $\delta^{15}N$ signature of TBWWTP POM is similar to that of clean coastal and oceanic POM measured in other studies (Cloern et al. 2002, Gartner et al. 2002) it is possible that high $\delta^{15}N$ values of *P. elongatus* in 'high impact' sites show a movement of $\delta^{15}N$-enriched sewage DIN into the pelagic food chain. Due to dilution effects on sewage DIC is it unlikely that sewage DIC could similarly significantly influence $\delta^{13}C$ levels of *P. elongatus*.

Increased sewage influence at sites close to the outflow appears to be reflected in a reduction in *P. elongatus* $\delta^{13}C$ signatures. This is likely to reflect the feeding patterns of *P. elongatus,* a filter feeder of water-column particulates (Morton and Miller 1968). Typical marine POM $\delta^{13}C$ signatures range from ~ –19‰ to –24‰ (Cloern et al. 2002, Evans et al. 2006, Piola et al. 2006, Vizzini and Mazzola 2006). Because TBWWTP SPOM carbon ($\delta^{13}C$ = -27.4‰ ± 0.76 S.E) is separated from this, it is likely that an increase in dietary intake of SPOM at sites close to the TBWWTP outflow is the cause of depleted $\delta^{13}C$ signatures of *P. elongatus* tissues in those areas.

Conclusions

The influence of sewage DIN $\delta^{15}N$ signatures in *C. maschalocarpum* at high impact sites, and the large range in $\delta^{15}N$ signatures between 'low impact' and 'high impact' sites suggest that *C. maschalocarpum* $\delta^{15}N$ signatures are particularly sensitive indicators of dissolved effluent availability. In addition, depleted $\delta^{13}C$ levels in the tissue of the benthic filter-feeder *P. elongatus* only in areas of high particulate concentrations adds to the body of evidence that suggests benthic filter-feeders are a

suitable measure of dispersal of terrestrially-derived POM. Notably, the low $\delta^{13}C$ values of POM released from this treatment plant facilitate this effect, and similarly, the ^{15}N-enriched nature of TBWWTP DIN enables its sewage DIN to be traced. Tracing studies of this nature require dissolved and particulate isotope source signatures distinct from those nutrient sources available to organisms in natural state environments.

Chapter 5 - General Discussion

Variability in $^{14}N/^{15}N$ fractionation in *Ulva* sp. as a function of light level during algal growth highlights the need for care in the design of impact assessments and trophic studies that use $\delta^{15}N$ as a tracer of nitrogen flow. In particular, chapter 2 highlights the need for care in the design of $\delta^{15}N$ studies

1) across depth gradients
2) that require comparisons of nitrogen loading or trophic linkages in situations where varying turbidity may affect the available light to algae.

Some studies of nitrogen loading may require modification to traditional mixing models that calculate percentage contributions of various potential nitrogen sources (Smit 2001, Umezawa et al. 2002, Lapointe et al. 2005). Where depth strata vary consistently in light availability, it may be plausible to modify traditional mixing models to account for depth dependent fractionation effects. Potentially, such modifications would require an understanding of how fractionation is likely to vary across a gradient of light levels, as a function of species, and the relative availability of different inorganic forms of nitrogen. Notably, water column turbidity may be increased by eutrophication, via increases in phytoplankton densities (Short and Wyllie-Echeverria 1996, Greve and Krause-Jensen 2005).

Studies of phytoplankton and terrestrial plants have shown considerable among-species variability in $^{14}N/^{15}N$ fractionation (Waser et al. 1998, Kolb and Evans 2003). However, the mechanisms that control $^{14}N/^{15}N$ fractionation in algae are poorly understood (Handley and Raven 1992, Waser et al. 1998). Research opportunities

exist firstly in developing a mechanistic understanding of $^{14}N/^{15}N$ fractionation in algae, and secondly in identifying how environment-driven fractionation differences are likely to vary across taxonomic groups and macroalgal morphologies. Because macroalgae differ in their physiology of uptake, assimilation and storage of nitrogen, it is likely that some species of macroalgae will fractionate differently than others (Wallentinus 1984, Waser et al. 1998). Differences in $\delta^{15}N$ levels of algal species living in close proximity to each other would suggest that this is the case in natural environments (Cloern et al. 2002, Vizzini et al. 2005). Species that fractionate more stably under different environmental conditions would be preferable species for $\delta^{15}N$ tracer studies. These species will generate tissue $\delta^{15}N$ ratios that are representative of the $\delta^{15}N$ ratios of their source nitrogen consistently over the physical gradients common in impact assessments and at freshwater/marine boundary points (Ellis et al. 2000, Huret et al. 2005). A mechanistic understanding of macroalgal nitrogen fractionation would aid in the selection of these species by researchers.

The ability of *Ulva* sp. to assimilate considerable quantities of nitrate in unreduced form (Naldi and Wheeler 1999), combined with the acclimatisation of this species to reducing heavy nitrate loads under low light conditions shown in chapter 3, may account for the comparatively low $^{14}N/^{15}N$ fractionation found for *Ulva* sp. in this study relative to those in studies of phytoplankton growth on nitrate (Waser et al. 1998, Needoba et al. 2003, Needoba et al. 2004). Phytoplankton show considerable fractionation effects under high nitrate and low light (Needoba and Harrison 2004), and there is evidence that the mechanism for fractionation during algal growth on nitrate is efflux of ^{15}N-enriched internal pools of nitrate when the N assimilation

process is rate limited by the energetic requirements of nitrate reduction (Wada and Hattori 1978, Needoba et al. 2004).

Further study on this mechanism and on the mechanism of fractionation during macroalgal growth on ammonium would require experimental facilities for keeping macroalgae alive in a closed system. In this manner it would be possible to completely control the level and $\delta^{15}N$ signature of seawater DIN and measure change in the $\delta^{15}N$ signature of this DIN. Analysis of this type would also require measurement of $\delta^{15}N$ signatures of intracellular pools of nitrogen to isolate the stage of nitrogen assimilation at which isotope discrimination occurs, e.g., see Needoba et al. (2004).

Chapter 2 shows that $\delta^{13}C$ levels in macroalgae may vary considerably over gradients of sewage influence and nutrient enrichment. The experimental portion of this chapter showed that light and nutrient limitation may affect algal $\delta^{13}C$ levels independently of source DIC $\delta^{13}C$ signatures. Experimental manipulation of nitrogen and light supply within levels found in natural environments generated differences in $\delta^{13}C$ signatures of ~ 11‰. Field measurements over a gradient of anthropogenic nutrient enrichment resulted in a range in algal $\delta^{13}C$ levels of ~ 6.7‰. One of the potential consequences of the large differences in $\delta^{13}C$ levels measured in this chapter relates to trophic studies that employ carbon isotopes in consumer tissues to deduce feeding patterns. The high variation in a macroalga shown here over short spatial and temporal scales suggests that considerable noise may be introduced into consumer feeding studies that extend over physical and chemical gradients that can affect algal $\delta^{13}C$ levels. However, environmental influences on $\delta^{13}C$ levels in natural algal populations extend

beyond the effects of light and nutrient limitation shown in this study. Algal $\delta^{13}C$ signatures can also be affected by turbulence (Finlay 2004), and vary on geographical scales possibly as a result of temperature differences (Wiencke and Fischer 1990, Smit 2001). Because of this, particular attention in food source study design should be paid to generating 'average' $\delta^{13}C$ values for food sources that are accurate over the spatial scales of the consumer's feeding range, and care should be exercised in the interpretation of results where the mean $\delta^{13}C$ ratios of possible food sources are closely spaced.

Chapter 4 illustrates the use of $\delta^{15}N$ signatures in a macroalga and grazer as a tracer of isotopically heavy sewage DIN from the dissolved fraction of sewage effluent, and $\delta^{13}C$ signatures in a filter-feeder as a tracer of sewage particulates. The successes of these tracing methods depend on the significant separation of effluent source signatures from nutrient sources in unimpacted environments. For effluent from the TBWWTP, macroalgal $\delta^{15}N$ ratios are a good proxy for dissolved effluent dispersal patterns because the $\delta^{15}N$ ratio of effluent DIN is considerably higher than that of the DIN generally available to macroalgae in unimpacted areas (Gartner et al. 2002, Bedard-Haughn et al. 2003). With reference to appendix 5, it should not be assumed that this effect is applicable to all tertiary wastewater treatment plants. The treatment plants at Moa Point and Hutt Valley appear to generate DIN $\delta^{15}N$ signatures within the range commonly associated with unimpacted areas (Rogers 1999, Bedard-Haughn et al. 2003, Rogers 2003, Savage and Elmgren 2004).

In contrast, $\delta^{13}C$ signatures would appear to be stable across wastewater treatment plants and treatments. The mean $\delta^{13}C$ signature across all plants and dates measured here (-25.85‰ ± 0.236 S.E.) would appear to be typically (but not always) lower than

$\delta^{13}C$ signatures for POM across a wide range of coastal and oceanic systems (Cloern et al. 2002, Evans et al. 2006, Piola et al. 2006, Vizzini and Mazzola 2006).

There are also few macroalgae that generate average $\delta^{13}C$ signatures lower than the $\delta^{13}C$ signatures for sewage particulates measured here (Raven et al. 2002). Generally these are macroalgae that are not able to utilize isotopically heavier HCO_3^- as a carbon source (Raven et al. 1995, Raven et al. 2002). Because of this, sewage POM is likely to generate more negative $\delta^{13}C$ signatures in the tissue of most marine organisms that consume it. Notably however, the difference between sewage POM and alternative sources of particulate carbon is likely to be less in areas affected by natural terrestrial sources of POM (Moore and Suthers 2005).

Patterns observed throughout this thesis highlight a particular need for greater understanding of the drivers of isotopic change in marine primary producers. An understanding of these drivers is required to interpret spatial and temporal variability in consumer isotope ratios. Studies that use isotope ratios to deduce patterns of enrichment and consumer feeding commonly examine the tissues of organisms with slow turnover times, and that feed over wide spatial scales (Gaston et al. 2004, Gaston and Suthers 2004, Hadwen and Arthington 2007). Where the isotopic composition of nutrient sources such as algae vary spatially and temporally, there is no guarantee that the isotopic composition of the organism is proportional to the mean isotopic values of its nutrient sources. For example, seasonal patterns in turnover of consumer tissues (Perga and Gerdeaux 2005), combined with seasonal patterns of isotopic change in algae (Cloern et al. 2002, Vizzini and Mazzola 2003) provide a mechanism for separation of consumer isotope ratios from the mean values of the food they have

consumed. Uneven spatial patterns in isotope ratios of a single food/nutrient source, combined with spatially uneven feeding patterns would similarly provide a mechanism for separation of consumer isotope ratios from the mean values of the food within their feeding range.

While temporal and spatial variability in algal isotope ratios can hinder interpretation of consumer isotope data, when understood it could potentially provide insight into small-scale spatial and temporal variability in ecological systems.

Appendices

Appendix 1 - Measurement of very low ammonium concentrations

In order to accurately measure the very low concentrations of ammonium present in scrubbed seawater it was necessary that I modified the methods of Koroleff (1983) by using a distilled water blank instead of a low-ammonium seawater blank. Because the deionised water blank was different in chemical composition (other than ammonium) from the samples being measured I compared the response of the distilled water to seawater in spectrophotometer absorbance tests. I generated three standard curves using distilled water, scrubber seawater and coastal (Goat Island) seawater with a gradient of artificial ammonium additions between 0 and 40μM. Parallel lines would indicate an identical linear spectrophotometer response to the increases of ammonium for all three blanks. The zero blank for all these measurements was made using distilled water with no added ammonia. Notably, on the day of this test Goat Island seawater was depleted (to near zero) in ammonium, and this is reflected in the similar y-intercepts of all three samples. While absorbance appears to increase slightly in distilled water samples when ammonium concentrations are high, the relationship appears stable at concentrations less than 20μM. (Fig. 6.1, Table 6.1).

Table 6.1 Regressions on ammonium standard curve with alternative blanks

Curve	Adjusted R^2	Estimate S.E.	Slope	Y intercept	Intercept S.E.	p
Deionised water	0.9999	0.0016	0.0156	-0.0017	0.0009	<0.0001
Scrubber seawater	0.9998	0.0028	0.0147	-0.0004	0.0016	<0.0001
Goat Island seawater	0.9997	0.0030	0.0144	0.0088	0.0018	<0.0001

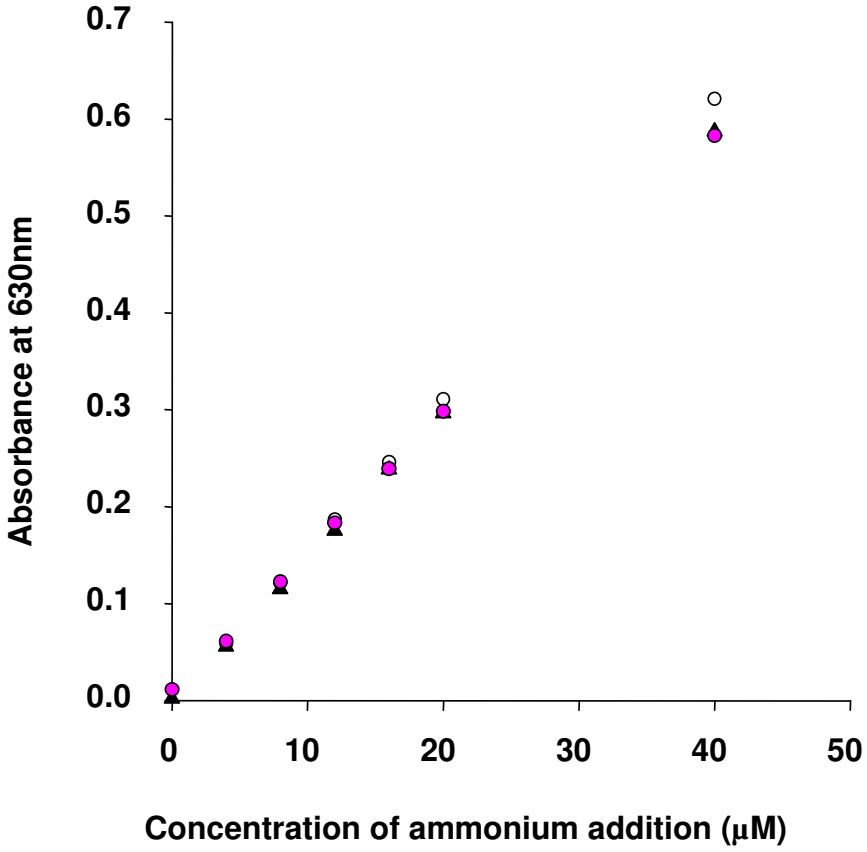

Figure 6.1 Spectrophotometer standard curves with ammonium additions to distilled water (white circles), scrubber seawater (black triangles), and Goat Island seawater (pink circles).

Appendix 2 – *Ulva* sp. tissue chlorophyll levels following experiment Chapter 3

At the conclusion of the experimental period of chapter 3, I measured tissue chlorophyll content, and took photos of *Ulva* sp. tissue from each treatment under magnification. To measure chlorophyll content I chopped the algal tissue and soaked it for 24 hours at 4°C in a solution of 4:1 methanol:dimethylsulphoxide (v:v)(Duncan and Harrison 1982). I removed the algae and measured absorbance of the solution at 650, 665 and 750nm. I converted the spectrophotometer measurements of absorbance to concentrations of chlorophyll *a* and *b* using formulae from Holden (1965).

The very low chlorophyll levels in the 'ambient light/no added nutrient' treatment (Table 7.1, Fig. 7.1 and 7.2) suggest that photosynthesis and growth in algae receiving this treatment was likely to have been limited by a lack of nitrogen (Healey 1973, Radmer and Kok 1977). The reduction in chlorophyll storage in nitrogen limited conditions is likely to have been caused by reduced chlorophyll production, and potentially via catabolism of chlorophyll compounds to supply nitrogen to other cellular processes (Bird 1982).

Table 7.1 Results of two-way ANOVA examining the influence of nutrient treatment and light treatment on chlorophyll (*a* + *b*) content in *Ulva* sp. (Chapter 3 - Day 14)

Response factor	Source of variation	*df*	SS	MS	F	p
Chlorophyll *a* + *b*	Nutrient	1	46.10	46.10	21.54	0.0017
	Light	1	191.5	191.5	89.48	<0.001
	Nutrient*light	1	0.056	0.056	0.0262	0.875
	Residuals	8	17.12	2.14		

Tukeys HSD post-hoc test between combinations of light and nutrient treatments.

Comparison		Difference	Lower	Upper	P adjusted
Ammonium/ Shade	Ammonium/ Light	4.057	0.2314	7.882	0.0381
Control / Light	Ammonium/ Light	-7.853	-11.679	-4.028	<0.001
Control / Shade	Ammonium/ Light	-4.070	-7.895	-0.2448	0.0375
Control / Light	Ammonium/ Shade	-11.91	-15.735	-8.085	<0.001
Control / Shade	Ammonium/ Shade	-8.127	-11.952	-4.3014	<0.001
Control / Shade	Control/ Light	3.783	-0.0419	7.609	0.0525

Figure 7.1

Figure 7.2

Appendix 3 – Within site and date variability in transplanted *Ulva* sp. δ13C, δ15N, %C and %N data

In order to gauge the between-plant variability in isotope values and C and N content at each mooring on each date, I sampled three whole plants from two sites on each of the three sampling dates. Sampling and analysis of algae was carried out following methods described in chapters 2 and 3. The sites for repeat sampling were selected to be representative of the highest and lowest available levels of sewage impact. Based on seawater nutrient concentrations, I judged site W3 as the most impacted site, and site E4 as the least impacted. Means and standard errors are presented in Table 8.1.

Table 8.1. Within site and date variability of $\delta^{13}C$, $\delta^{15}N$, %C and %N in transplanted *Ulva* sp. at two sites from the mooring array situated around the TBWWTP as described in chapter 2. Shown are the figures for the single (whole plant) sample used in statistical analyses, a mean value generated from individual analysis of three whole plants from the same site and date, and the standard error of that mean. The sites selected, W3 and E4, are representative of a high impact and low impact site respectively.

Site **E4** (surface)

Nov	%C	%N	δ13C	δ15N
Single sample	30.38	1.245	-22.185	7.19
Triplicate mean	31.67667	1.331667	-21.1283	7.43
Triplicate standard error	0.85861	0.045674	0.576602	0.187705
Jan				
Single sample	31.86	1.45	-19.495	10.285
Triplicate mean	31.82	1.55	-19.465	9.428333
Triplicate standard error	0.035119	0.076376	0.032532	0.486624
March				
Single sample	37.175	3.18	-17.14	7.725
Triplicate mean	35.80833	3.243333	-17.08	7.158333
Triplicate standard error	0.701536	0.053645	0.147422	0.324786

Site **W3** (surface)

Nov	%C	%N	δ13C	δ15N
Single sample	34.615	2.07	-19.045	16.765
Triplicate mean	35.17167	2.173333	-19.3817	18.505
Triplicate standard error	0.401002	0.053645	0.168333	1.575471
Jan				
Single sample	35.495	2.225	-13.1	22.21
Triplicate mean	34.58167	2.458333	-14.4167	21.98667
Triplicate standard error	0.548105	0.137184	0.677208	0.323024
March				
Single sample	35.145	2.945	-13.415	16.98
Triplicate mean	36.43167	2.965	-13.8717	16.39333
Triplicate standard error	0.643495	0.10153	0.414893	0.673457

Table 8.1. continued

Site **E4** (4m depth)

Nov	%C	%N	δ13C	δ15N
Single sample	41.075	2.655	-19.13	7.215
Triplicate mean	36.19167	2.351667	-19.0933	7.005
Triplicate standard error	2.466118	0.154389	0.274853	0.156072
Jan				
Single sample	29.87	1.22	-17.13	7
Triplicate mean	29.12333	1.24	-18.01	6.983333
Triplicate standard error	0.373333	0.030551	0.448442	0.101379
March				
Single sample	37.51	4.12	-16.35	7.205
Triplicate mean	34.83667	3.64	-16.6	6.485
Triplicate standard error	1.339469	0.246847	0.180278	0.367163

Site **W3** (4m depth)

Nov	%C	%N	δ13C	δ15N
Single sample	30.11	1.9	-20.85	8.895
Triplicate mean	32.455	1.975	-21.55	9.5975
Triplicate standard error	1.914684	0.061237	0.35473	0.573589
Jan				
Single sample	34.685	1.37	-18.94	11.82
Triplicate mean	35.045	2.156667	-18.08	11.77333
Triplicate standard error	0.757809	0.54364	0.502394	0.146211
March				
Single sample	32.725	2.89	-15.43	9.935
Triplicate mean	36.10833	3.18	-16.2933	8.295
Triplicate standard error	1.703693	0.145717	0.496532	0.830226

Appendix 4 – Nitrogen content of *Carpophyllum maschalocarpum* in the vicinity of the TBWWTP

To examine the effect of sewage nitrogen enrichment on the nitrogen status of the macroalga *Carpophyllum maschalocarpum* I examined tissue nitrogen content (%N) at a gradient of sites at increasing distances from the TBWWTP as detailed in chapter 5. I used the same tissue samples and analysis to collect %N data that I employed to collect $\delta^{13}C$ and $\delta^{15}N$ measurements in that chapter.

Patterns of nitrogen enrichment in *C. maschalocarpum* did not appear to follow general patterns of sewage dispersal. Rather, *C. maschalocarpum* %N levels appeared elevated at low-impact sites to the east of the outflow (compare Fig. 9.1 with Fig. 2.4).

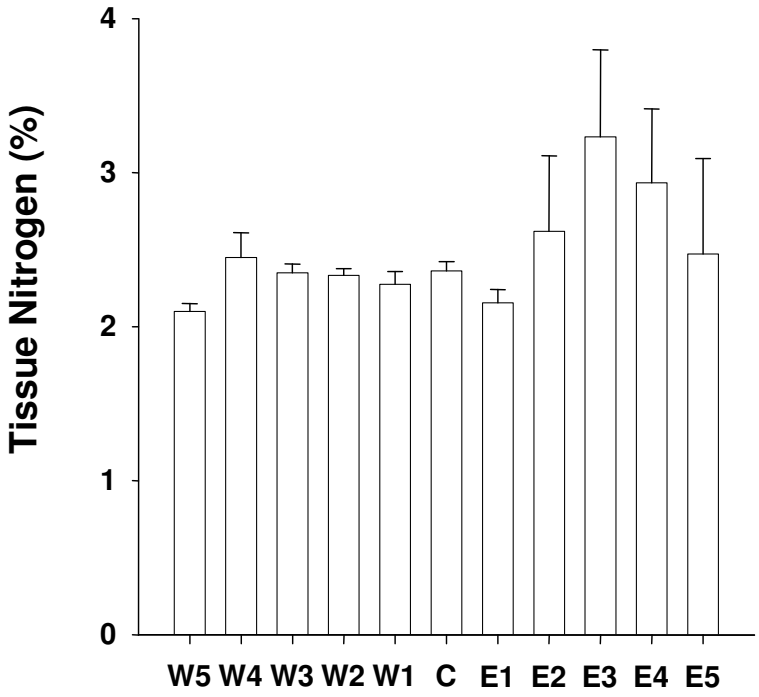

Figure 9.1. Time averaged nitrogen content of *C. maschalocarpum* tissue by site. Site marked C on the x-axis represents the sampling site at the outflow point to the TBWWTP, sites marked W1-W5 are at increasing distances to the west of the outflow, E1-E5 at increasing distances to the East of the outflow. Error bars +/- 1 S.E.

Appendix 5 –Variability in sewage effluent $\delta^{13}C$ and $\delta^{15}N$ signatures

To test whether values of source $\delta^{13}C$ and $\delta^{15}N$ signatures found in sewage effluent varied considerably between treatment plants, I sampled particulate $\delta^{13}C$ and $\delta^{15}N$ and DIN concentration and $\delta^{15}N$ signatures concurrently at two further tertiary processing plants in the Wellington area (at Moa Point and Hutt Valley). Staff from these facilities collected samples at ~9am on sampling days. Methods for my sampling and sample processing of effluent from these additional facilities are identical to those for TBWWTP detailed in chapter 2 (for dissolved effluent constituents) and chapter 5 (for particulates). I used 2-way ANOVAs to test the effects of treatment, treatment plant and their interaction on the DIN concentrations and $\delta^{15}N$ signatures of dissolved effluent, and also the $\delta^{13}C$ and $\delta^{15}N$ signatures of effluent POM. DIN concentration and DIN $\delta^{15}N$ data were log-transformed prior to ANOVA analysis to improve homogeneity of variances.

DIN concentration and $\delta^{15}N$ signatures of sewage DIN varied among treatment plants, treatment processing, and there was a significant interaction between these two factors (Table 10.1, Fig. 10.1). Overall, tertiary processing reduced DIN and increased $\delta^{15}N$, though the size of the tertiary processing effect varied greatly among treatment plants (i.e., the likely source of the significant interaction). $\delta^{15}N$ signatures of DIN were always higher than those for POM N from the same treatment plant. $\delta^{13}C$ signatures of sewage POM were not significantly separated either between treatments or between treatment plants (Table 10.2, Fig. 10.2).

Figure 10.1

Figure 10.2

Notably, $\delta^{15}N$ signatures of DIN in tertiary processed effluent discharged from both Moa Point and Hutt Valley plants during the period of my sampling were within the range of DIN $\delta^{15}N$ signatures commonly associated with 'clean' coastal areas (Spies et al. 1989, Rogers 1999, Smit 2001, Gartner et al. 2002, Savage et al. 2002, Bedard-Haughn et al. 2003, Savage et al. 2004; and see Table 1.2). The similarity of sewage DIN $\delta^{15}N$ signatures leaving these facilities and 'clean' coastal nitrogen signatures is potentially inconvenient for researchers/managers wishing to trace dispersal of dissolved nitrogen from the Hutt Valley and Moa Point (and other similar) facilities using $\delta^{15}N$ signatures in primary producers. Primary producer tissue generated in areas impacted by the Moa Point and Hutt Valley outflows during the sampling period are likely to have $\delta^{15}N$ signatures similar to primary producers from 'clean' areas.

The small increase in DIN $\delta^{15}N$ signatures between primary and tertiary processed effluent at the Moa Point and Hutt Valley WWTPs matched low nitrogen removal at these treatment facilities. The reduction and oxidation processes during sewage secondary and tertiary processing facilitate the removal of nitrogen from the effluent in the form of NH_4 and N_2 gases. Fractionation occurs during these processes generating heavier DIN $\delta^{15}N$ signatures in the remaining dissolved (tertiary processed) nitrogen pool (Macko and Ostrom 1994, Bedard-Haughn et al. 2003). Given the similarity of both DIN $\delta^{15}N$ signatures and DIN concentration in primary effluent at all facilities I would suggest that the higher $\delta^{15}N$ signature of TBWWTP tertiary DIN is a reflection of more efficient N-removal processes and hence greater fractionation of the sewage DIN pool at this facility.

Table 10.1. ANOVA analysis of sewage dissolved inorganic nitrogen (DIN) concentration change and DIN $\delta^{15}N$ change as a result of tertiary processing at three wastewater treatment plants

Response factor	Source of variation	*df*	SS	MS	F	p
Log (DIN concentration)	Plant	2	0.685	0.342	51.77	<0.001
	Treatment	1	1.085	1.085	164.1	<0.001
	Plant* Treatment	2	1.029	0.514	77.80	<0.001
	Residuals		0.079	0.007		
Response factor	Source of variation	*df*	SS	MS	F	p
Log ($\delta^{15}N$ (DIN))	Plant	2	0.334	0.167	22.79	<0.001
	Treatment	1	0.336	0.336	45.80	<0.001
	Plant* Treatment	2	0.147	0.073	9.99	0.003
	Residuals		0.088	0.007		

Table 10.2. ANOVA analysis of sewage POM $\delta^{13}C$ and $\delta^{15}N$ change as a result of tertiary processing at three wastewater treatment plants

Response factor	Source of variation	*df*	SS	MS	F	p
Particulate $\delta^{13}C$	Plant	2	1193.1	596.5	1.996	0.179
	Treatment	1	3.1	3.1	0.010	0.921
	Plant* Treatment	2	3.3	1.7	0.006	0.995
	Residuals	12	3586.2	298.8		
Response factor	Source of variation	*df*	SS	MS	F	p
Particulate $\delta^{15}N$	Plant	2	98.04	49.02	5.002	0.026
	Treatment	1	8.067	8.067	0.823	0.382
	Plant* Treatment	2	6.789	3.394	0.346	0.714
	Residuals	12	117.6	9.800		

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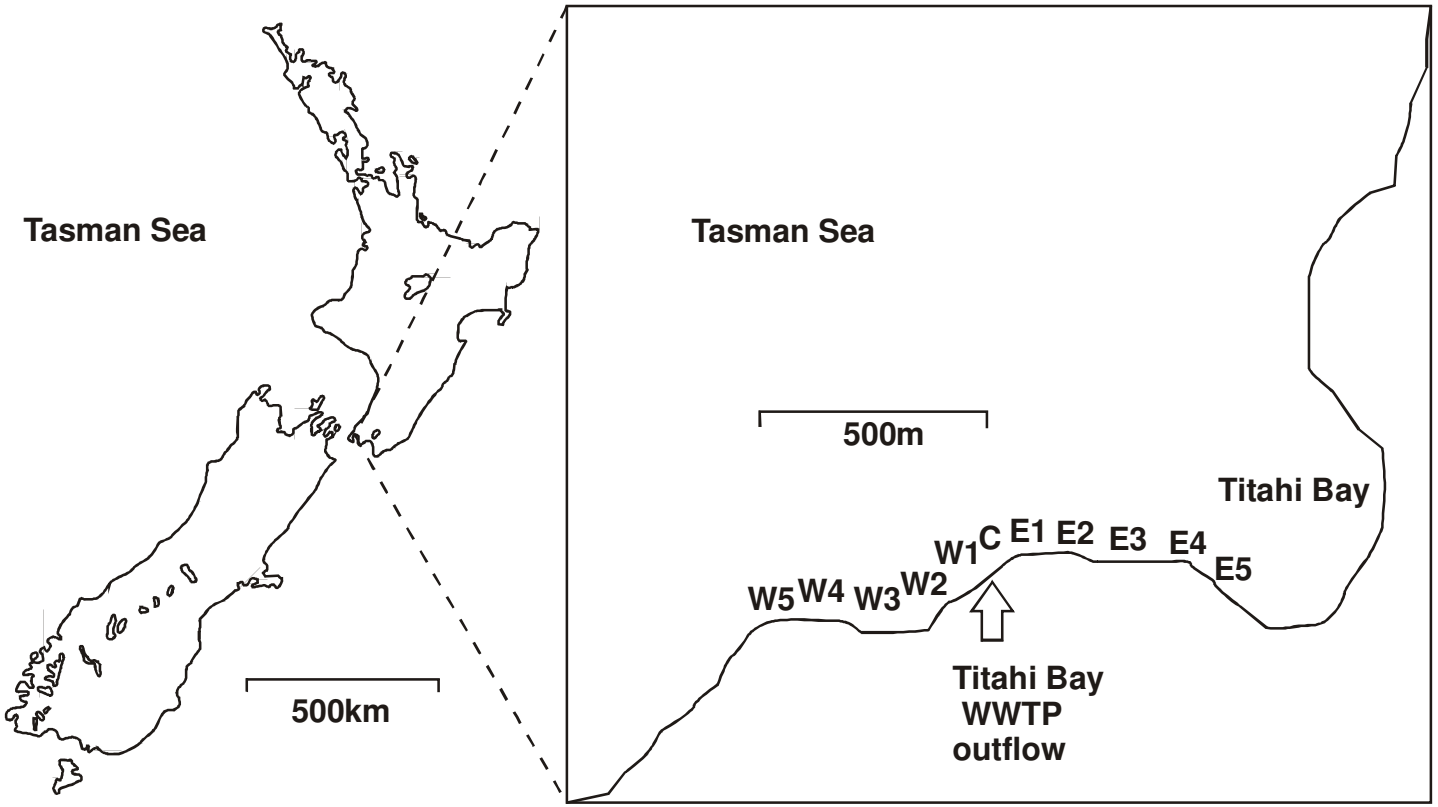


Figure 2.1
Location of field assessment of sewage effluent. Left panel gives location of sampled region within New Zealand, right panel shows the spatial arrangement of sites located ~50 m off the shoreline to the east and west of the outflow pipe of the Titahi Bay wastewater treatment plant. Sites are labelled sequentially to reflect distance from the discharge site (C), to the east (generally upcurrent) or to the west (downcurrent).

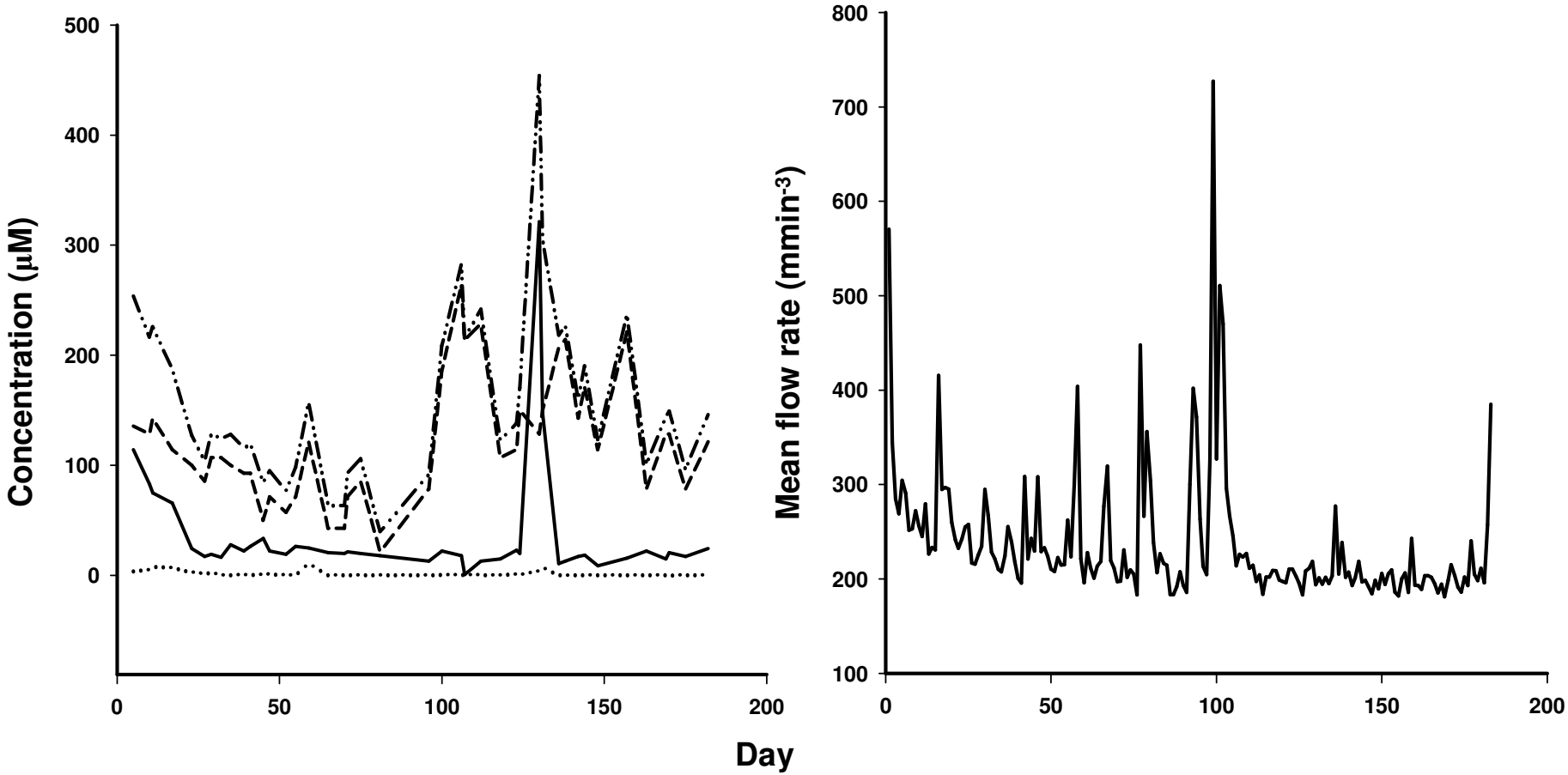


Figure 2.2
'**A'** Concentration of nitrogen ions in effluent leaving the Titahi Bay WWTP over the period of the field assessment.
Lines from bottom: dotted line gives nitrite concentration, dashed line gives nitrate concentration, solid line gives ammonia/ammonium concentration. Dotted/dashed line (top) gives total inorganic nitrogen (TIN).
'**B**' Flow rate of effluent leaving Titahi Bay WWTP over the period of the field assessment. Data courtesy of Hadley Bond, Porirua City Council.

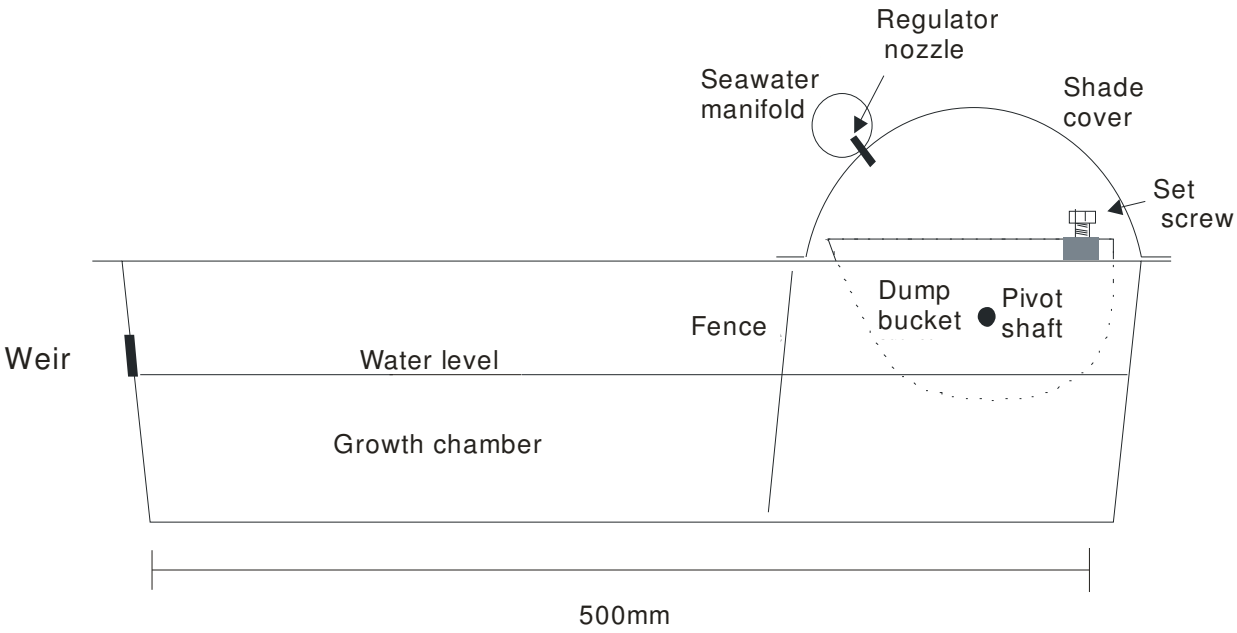


Figure 2.3. Diagram of an individual algal growth chamber, a component of the outdoor chemostat used to manipulate nutrients and light under turbulent flow conditions.

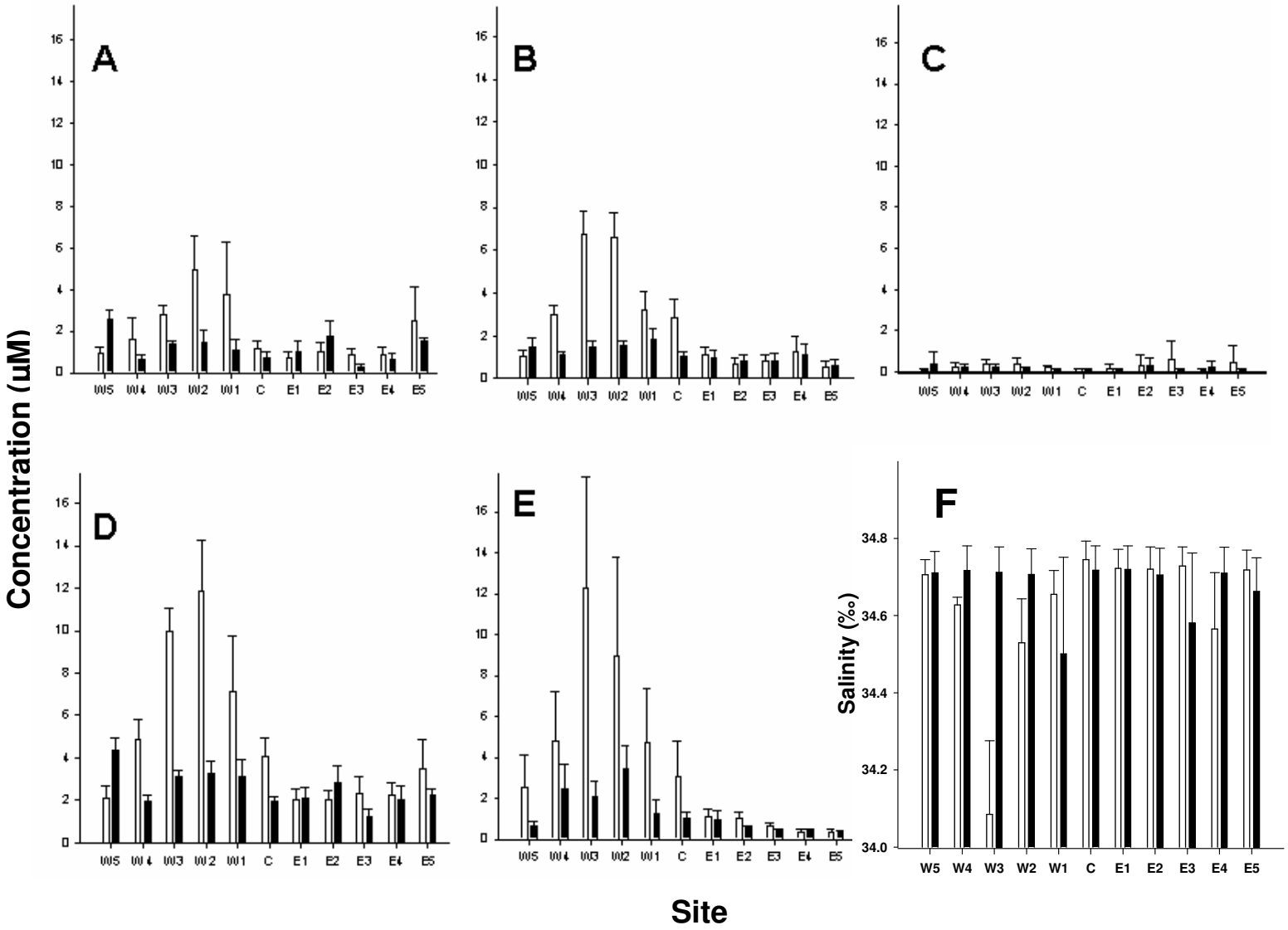


Figure 2.5. Concentrations of Ammonium (**A**), Nitrate (**B**),Nitrite (**C**), TIN (**D**), phosphate (**E**) ions, and salinity (**F**) in seawater sampled at surface (unshaded bars) and 4m depth (shaded bars) for an array of 11 sites near the Titahi Bay WWTP during summer 2004-2005.
Given are mean concentrations +/- S.E. n = 5.

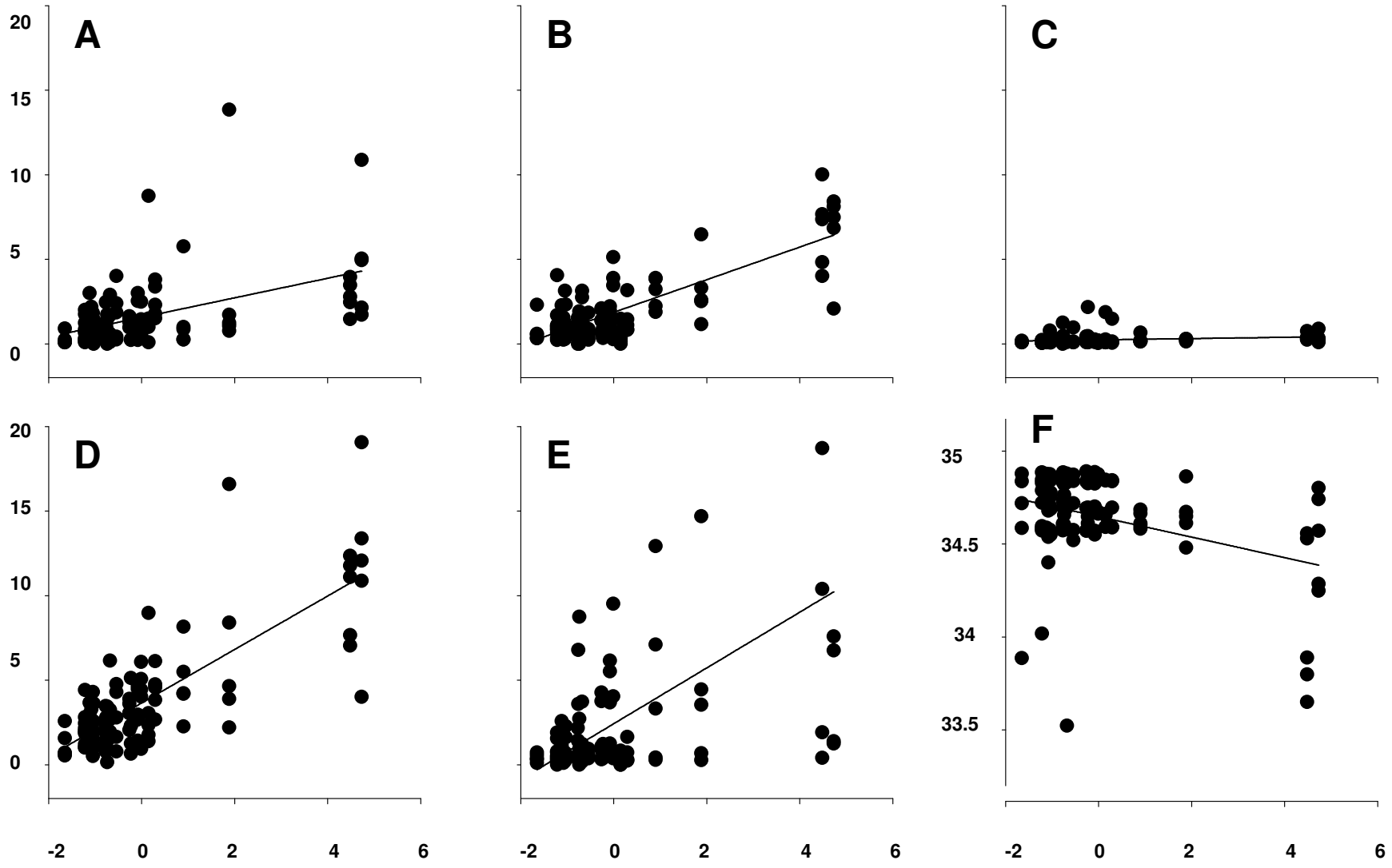


Figure 2.6. Concentrations of Ammonium (**A**), Nitrate (**B**), Nitrite (**C**), TIN (**D**), phosphate (**E**) ions, and salinity (**F**) in seawater samples, regressed against first principle component scores (PC1) for sewage influence for the site at which they were taken.
Plot A, $r^2 = 0.2278$, Plot B, $r^2 = 0.6341$, Plot C, $r^2 = 0.0340$, Plot D, $r^2 = 0.6170$,Plot E, $r^2 = 0.3265$, Plot F, $r^2 = 0.1357$.

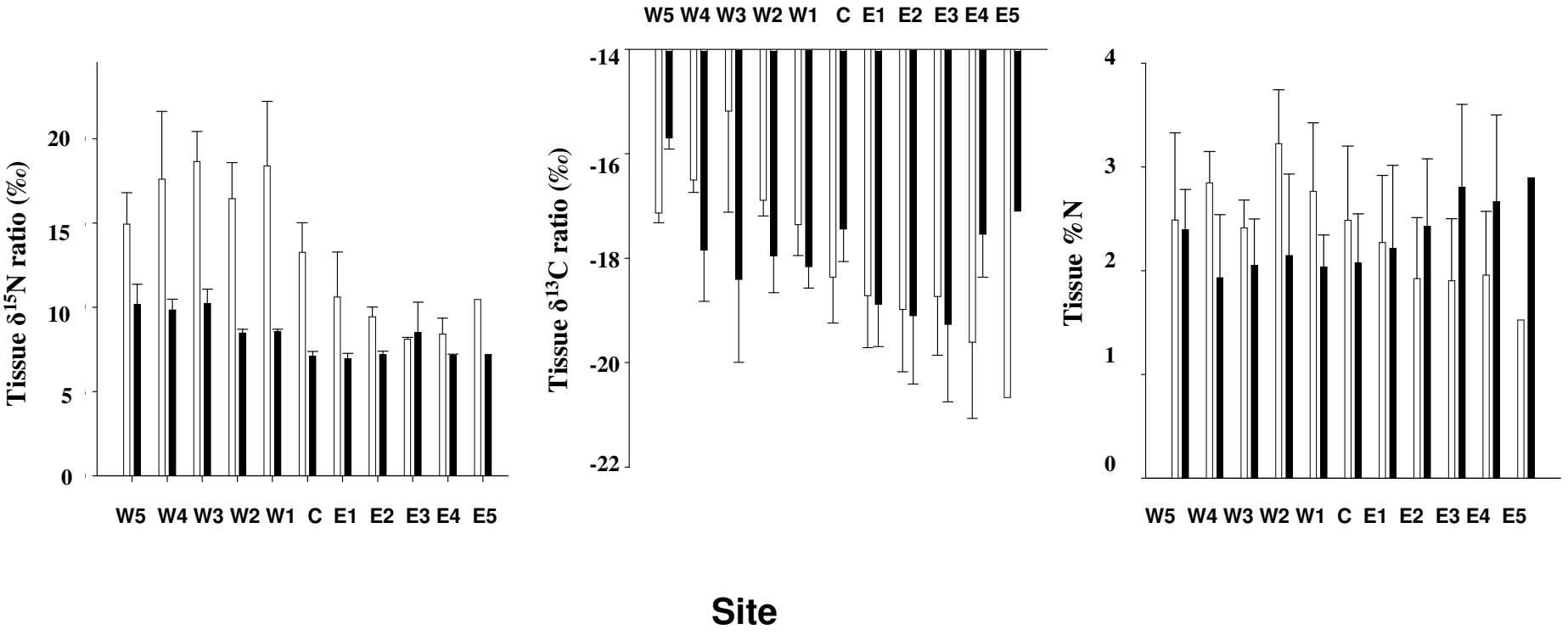


Figure 2.7. **A**. Time-averaged means of $\delta^{13}C$ ratios of *Ulva* sp. tissue taken from all sites. **B**. Time-averaged means of $\delta^{13}C$ ratios of *Ulva* sp. tissue taken from all sites. **C**. Time-averaged means of tissue nitrogen content for *Ulva* sp. tissue taken from all sites. Unshaded bars represent surface sites, shaded bars are from sites at 4 metres depth. n= 3, error bars +/- S.E. January and March replicates were lost for site E5, n=1 for this site.

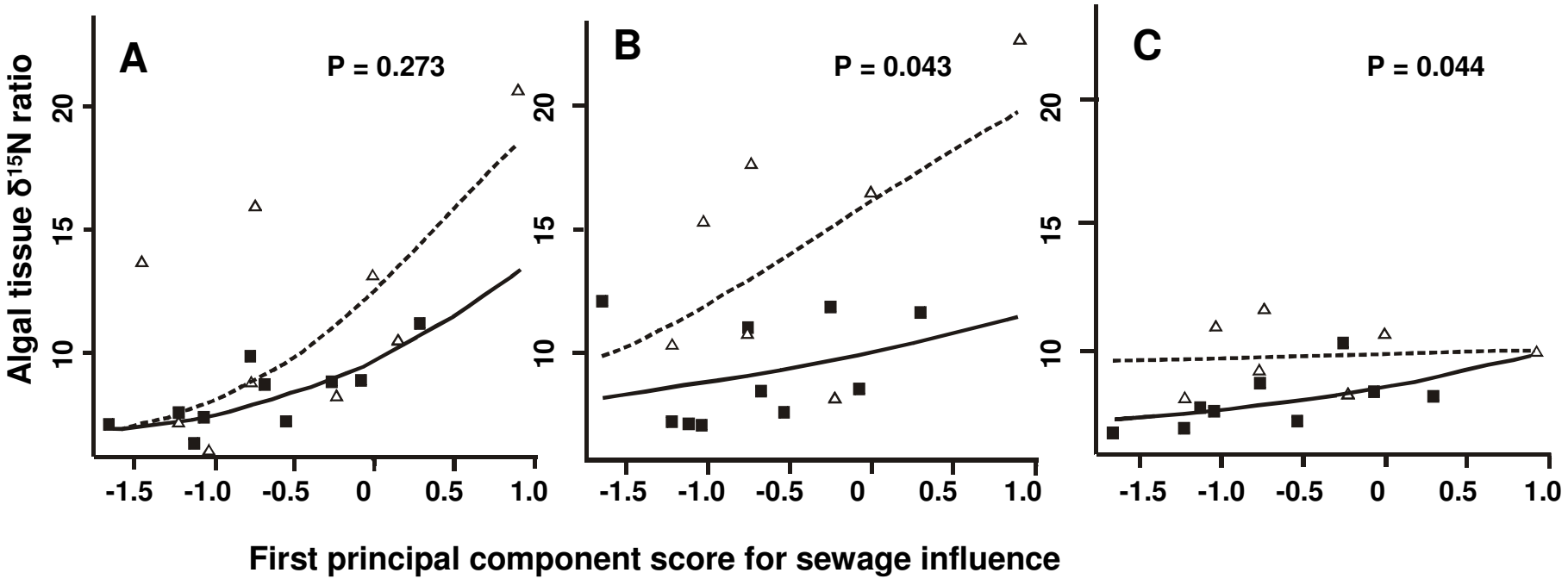


Figure 2.8. Response of tissue $\delta^{15}N$ signatures to variation in sewage effluent concentration estimated from the first principal component score of a PCA (see methods). Fitted lines were generated from a logistic regression (see Table 2). Dashed line fitted to white triangles represents the predicted response of *Ulva* sp. tissue to sewage concentrations near the seawater surface while solid line fitted to black squares represents the predicted response of *Ulva* sp. to sewage concentrations at 4m depth. P-values given in each panel evaluate the null hypothesis that the responses of *Ulva* sp. to sewage concentrations are the same at the surface and at depth. Relationships are evaluated separately for three sampling periods (**A**) October-November (**B**) November-January, (**C**) January-March.

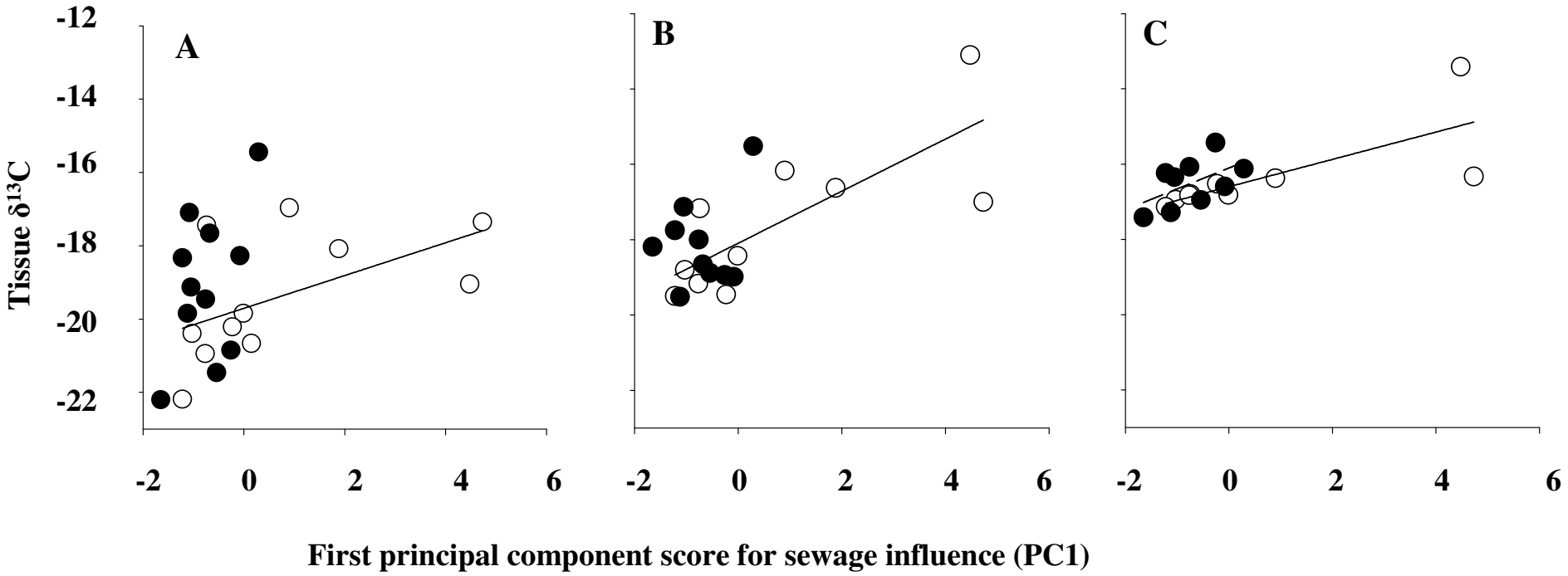


Figure 2.9. Regression of tissue $\delta^{13}C$ ratios of whole *Ulva* sp. individuals collected from around the TTBWWTP against first principal component (PC1) score for sewage influence for the site at which they were collected. Plots A, B and C show $\delta^{13}C$ ratios from algae collected in November, Janurary and March, respectively. White circles show surface sites, black circles show sites at 4 metres depth. Solid lines are used for significant ($p < 0.05$) regressions of surface sites, dashed lines are used for significant regressions of 4m sites.
Plot **A**, regression surface; $r^2 = 0.2255$
Plot **B,** regression surface; $r^2 = 0.5965$
Plot **C**, regression surface; $r^2 = 0.5502$, regression 4m; $r^2 = 0.2917$

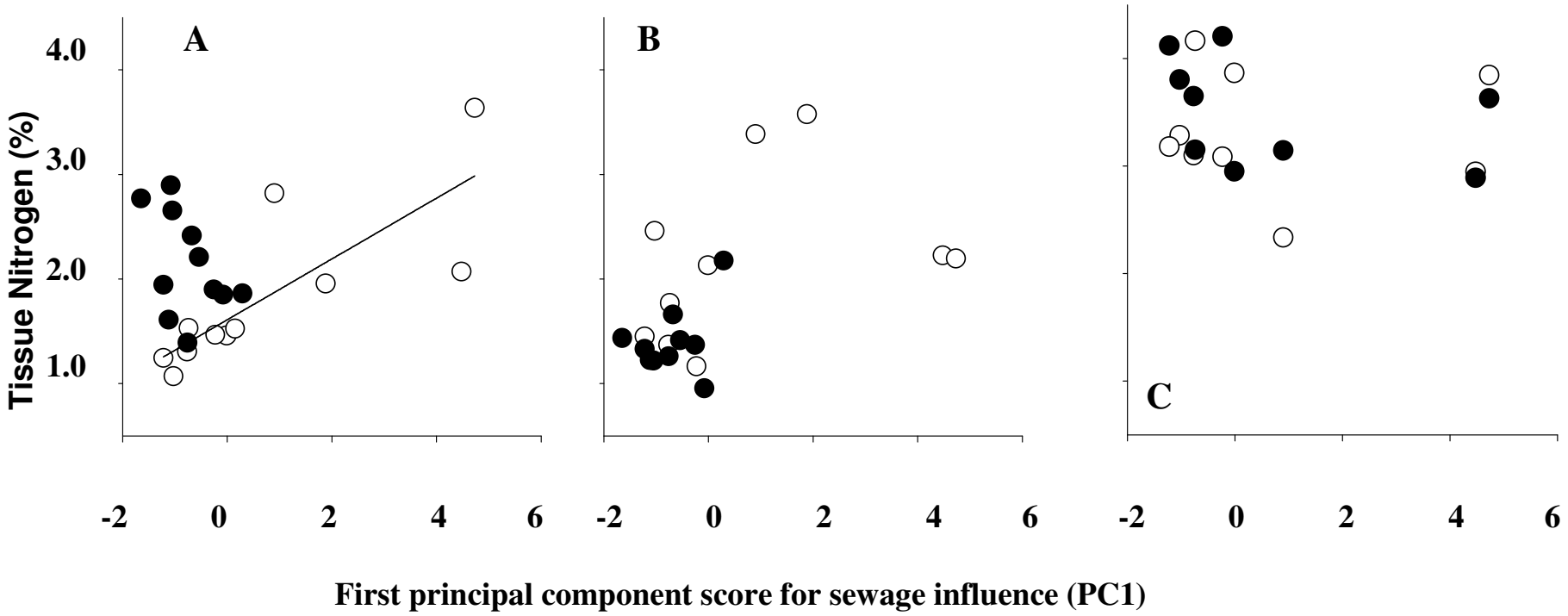


Figure 2.10. Regression of tissue nitrogen content of whole Ulva sp. individuals collected from around the TBWWTP against first principal component (PC1) score for sewage influence for the site at which they were collected. Plots **A**, **B** and **C** show tissue nitrogen content from algae collected in November, January and March, respectively. White circles show surface sites, black circles show sites at 4metres depth. Solid lines are used for significant ($p < 0.05$) regressions of surface sites, dashed lines are used for significant regressions of 4m sites.
Plot **A**, regression surface; $r^2 = 0.6310$

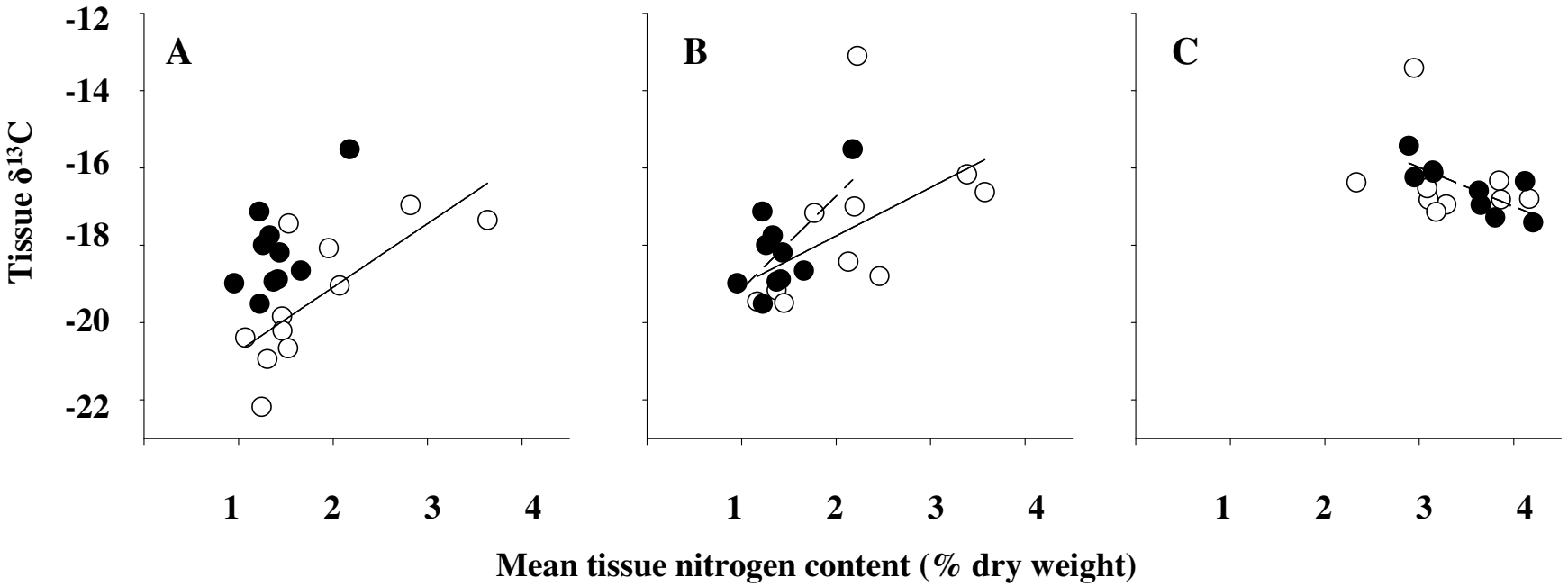


Figure 2.11. Regression of tissue $\delta^{13}C$ against tissue nitrogen content from whole *Ulva* sp. individuals collected from around the TTBWWTP. Plot A, B and C show data from algae collected in November, January and March, respectively. White circles show surface sites, black circles show sites at 4metres depth. Solid lines are used for significant ($p < 0.05$) regressions of surface sites, dashed lines are used for significant regressions of 4m sites.
Plot **A**, regression surface; $r^2 = 0.6310$
Plot **B,** regression surface; $r^2 = 0.2609$, regression 4m; $r^2 = 0.4546$
Plot **C**, regression 4m; $r^2 = 0.6087$

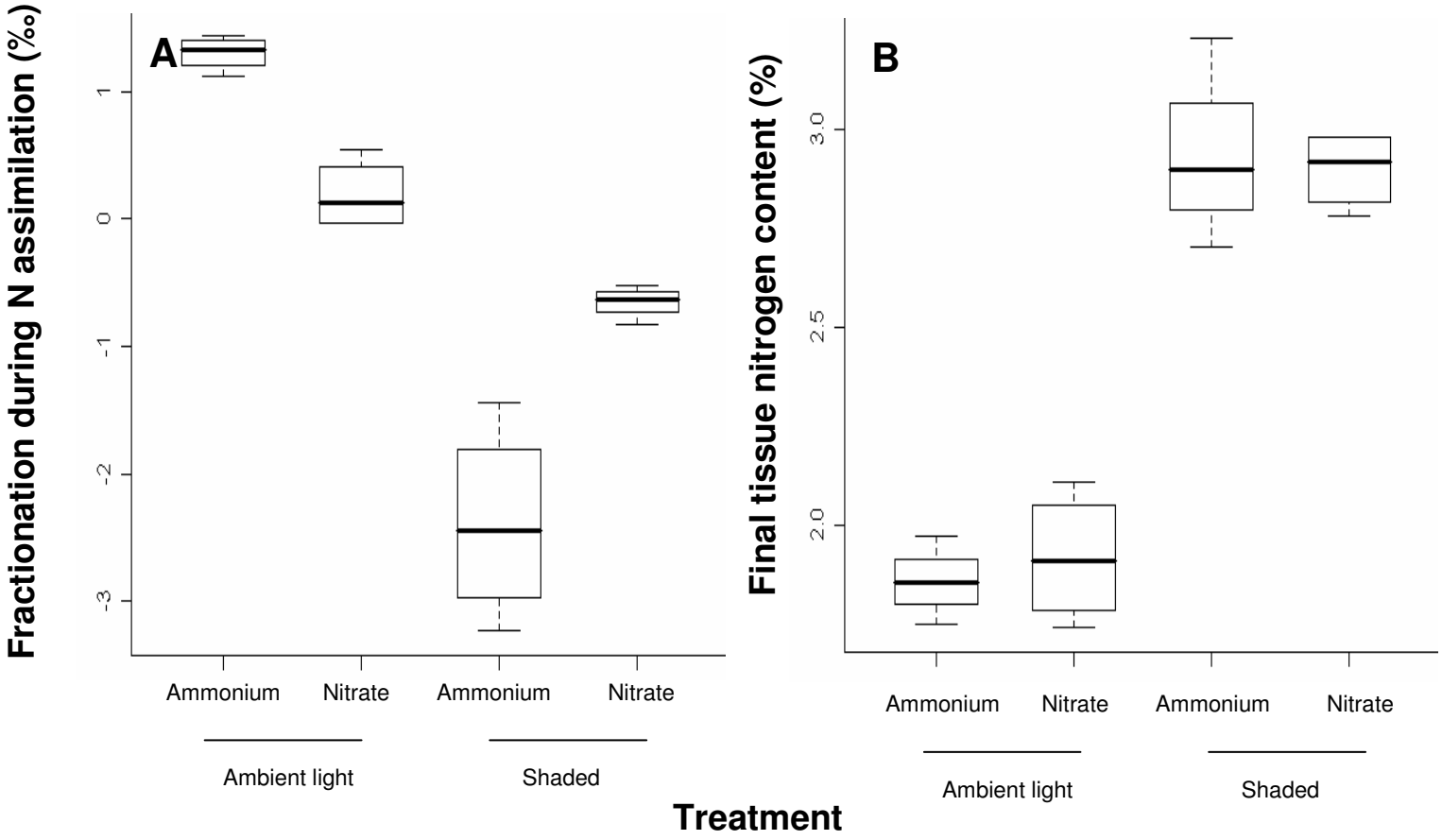


Figure 2.12. Effects of light availability and nutrient source on fractionation of nitrogen (**A**) and tissue nitrogen content (**B**) of *Ulva* sp. from the $\delta^{15}N$ laboratory experiment (Experiment 1). Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment.

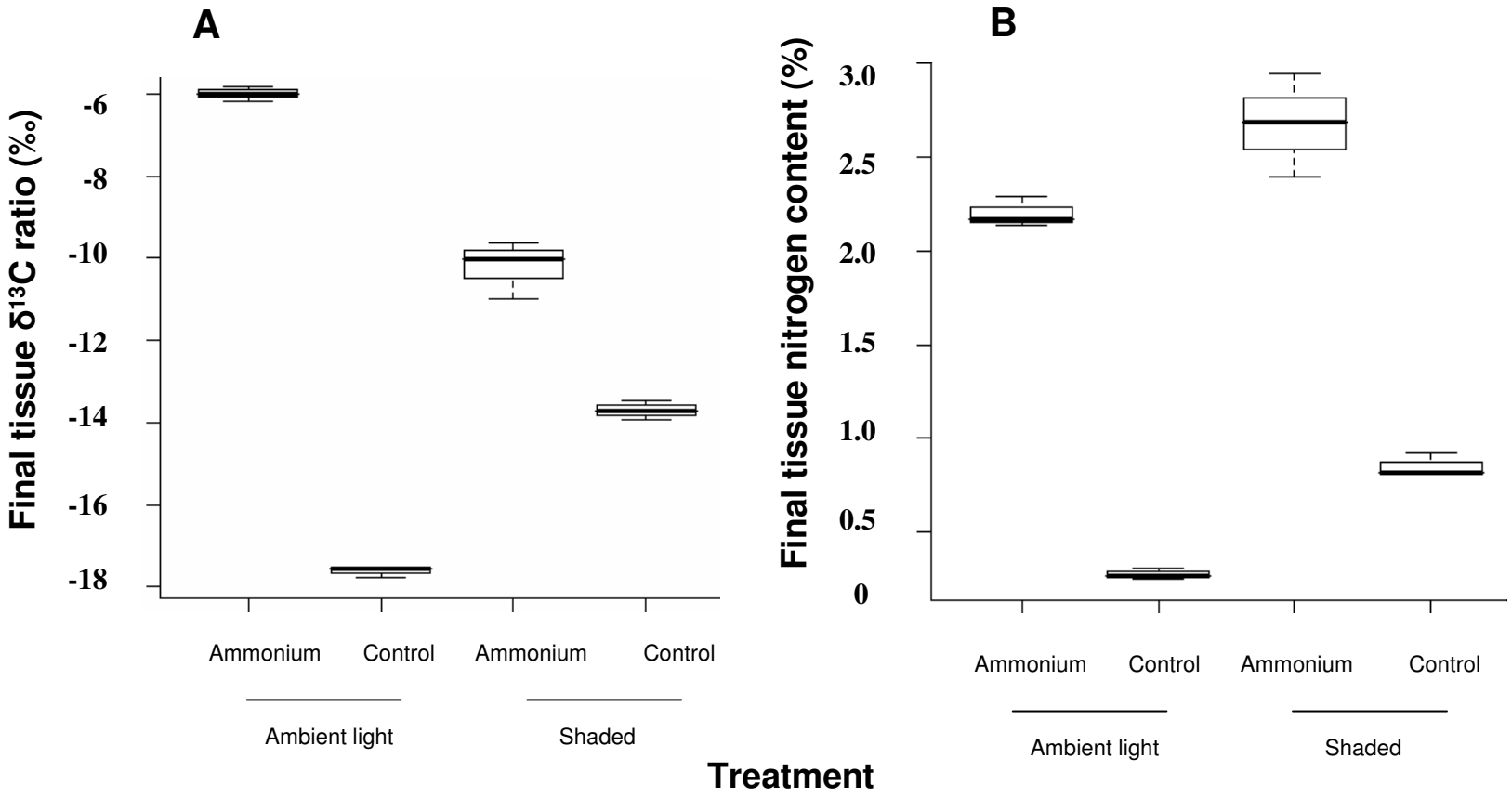


Figure 2.13. Effects of light and nutrient availability on fractionation Final tissue $\delta^{13}C$ ratio (‰) (**A**) and tissue nitrogen content (**B**) of *Ulva* sp. from the $\delta^{13}C$ laboratory experiment (experiment 2). Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment.

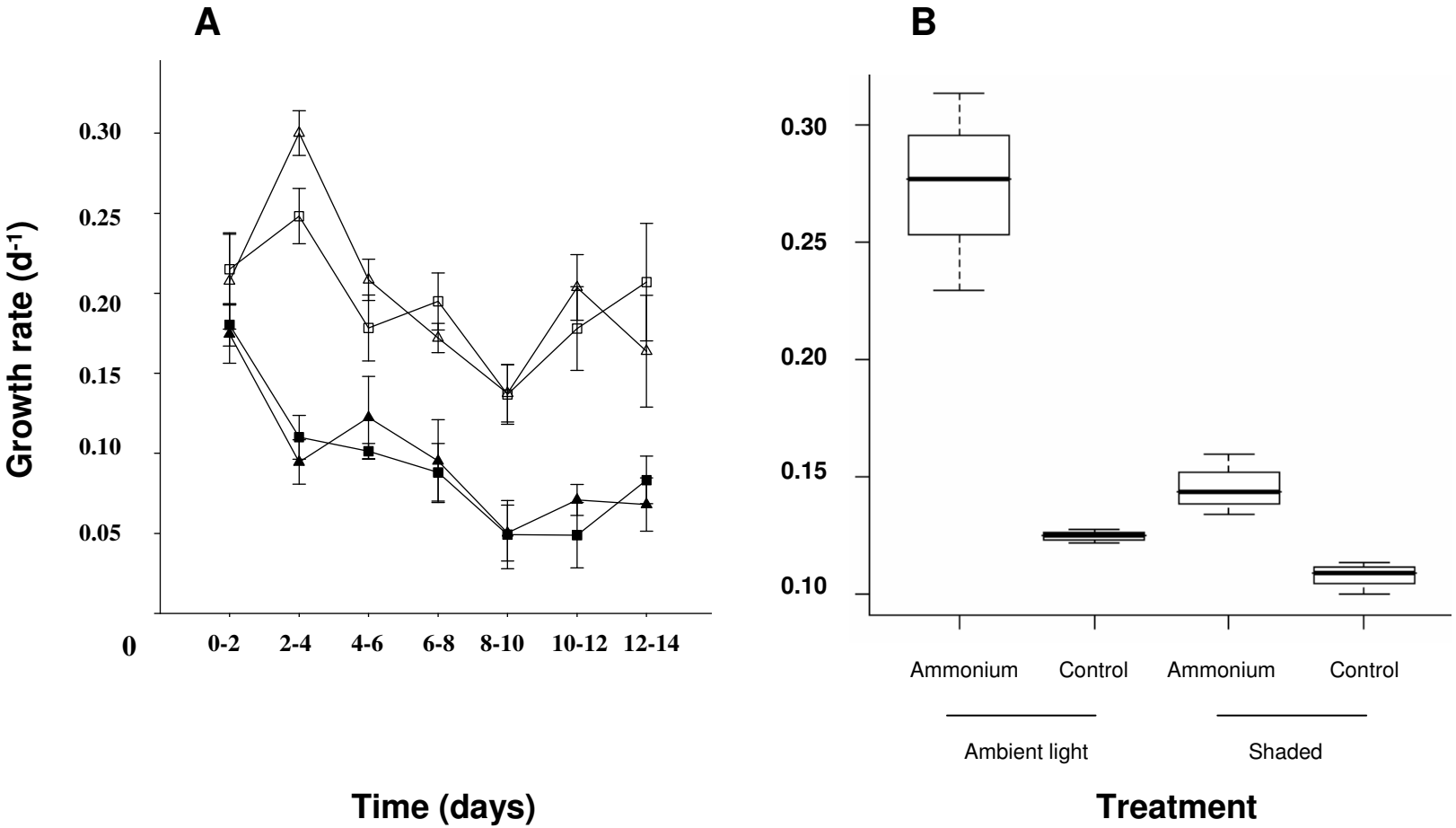


Figure 2.14. Effects of light and nutrient availability on growth rate of *Ulva* sp in $\delta^{15}N$ laboratory experiment (**A)** and $\delta^{13}C$ laboratory experiment (**B**). Growth rates for **A** were measured every two days, growth rates for **B** were averaged over the length of the experiment.

A. Triangles are chambers receiving ammonium as a nitrogen source, squares are chambers receiving ammonium as a nitrogen source. Shaded chambers are represented by shaded symbols, unshaded chambers are represented by unshaded symbols. Errors bars +/- 1 S.E n=4 for each point.

B. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment.

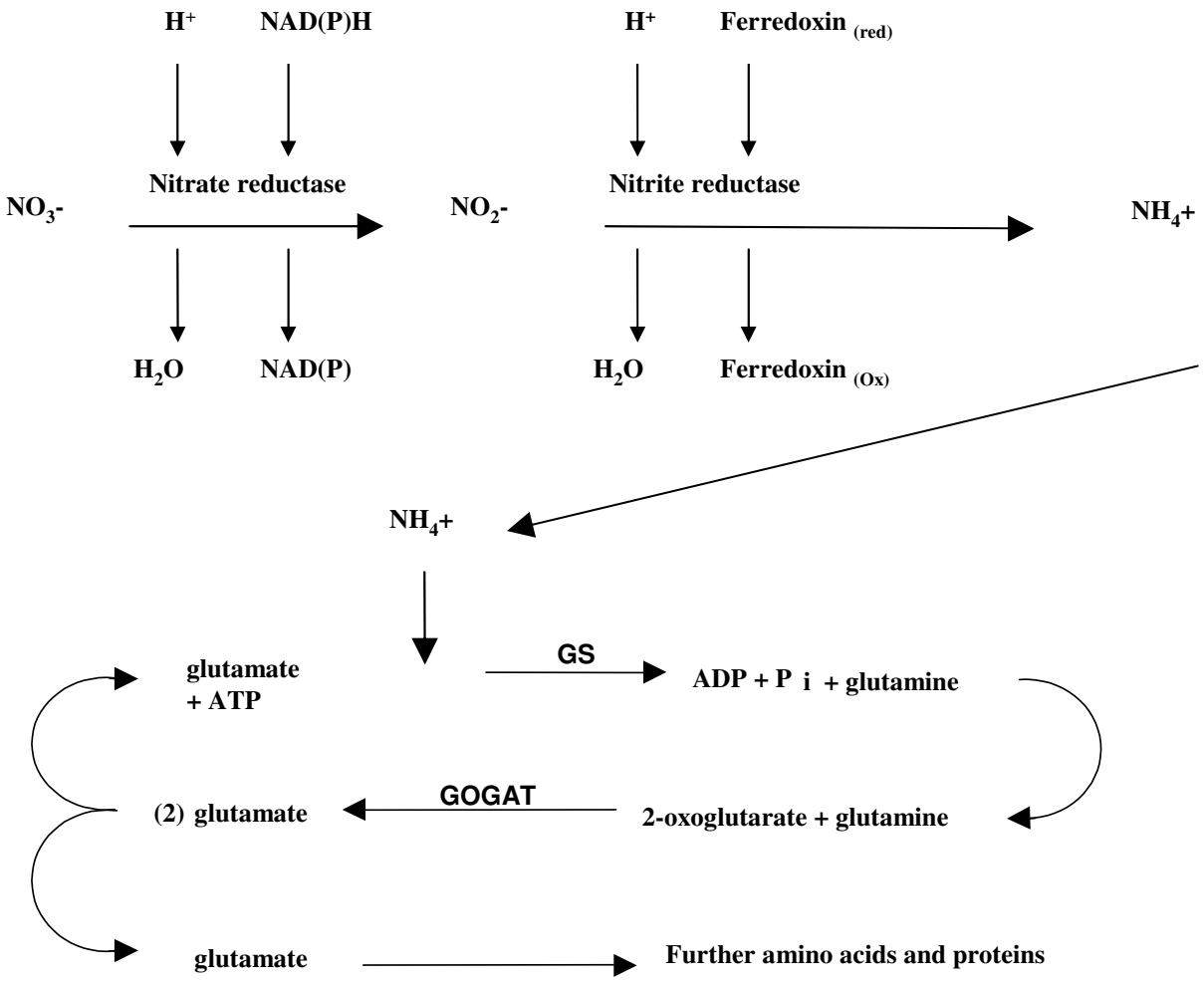


Figure 3.1. Representation of nitrate reduction and assimilation in algae. Nitrate (NO_3-) is reduced to nitrite (NO_2-) and then ammonium (NH_4+), then converted to glutamine and subsequent amino acids via the Glutamine synthetase/glutamine : 2-oxoglutarate aminotransferase (GS/GOGAT) pathway. Figure modified from Syrett (1981)

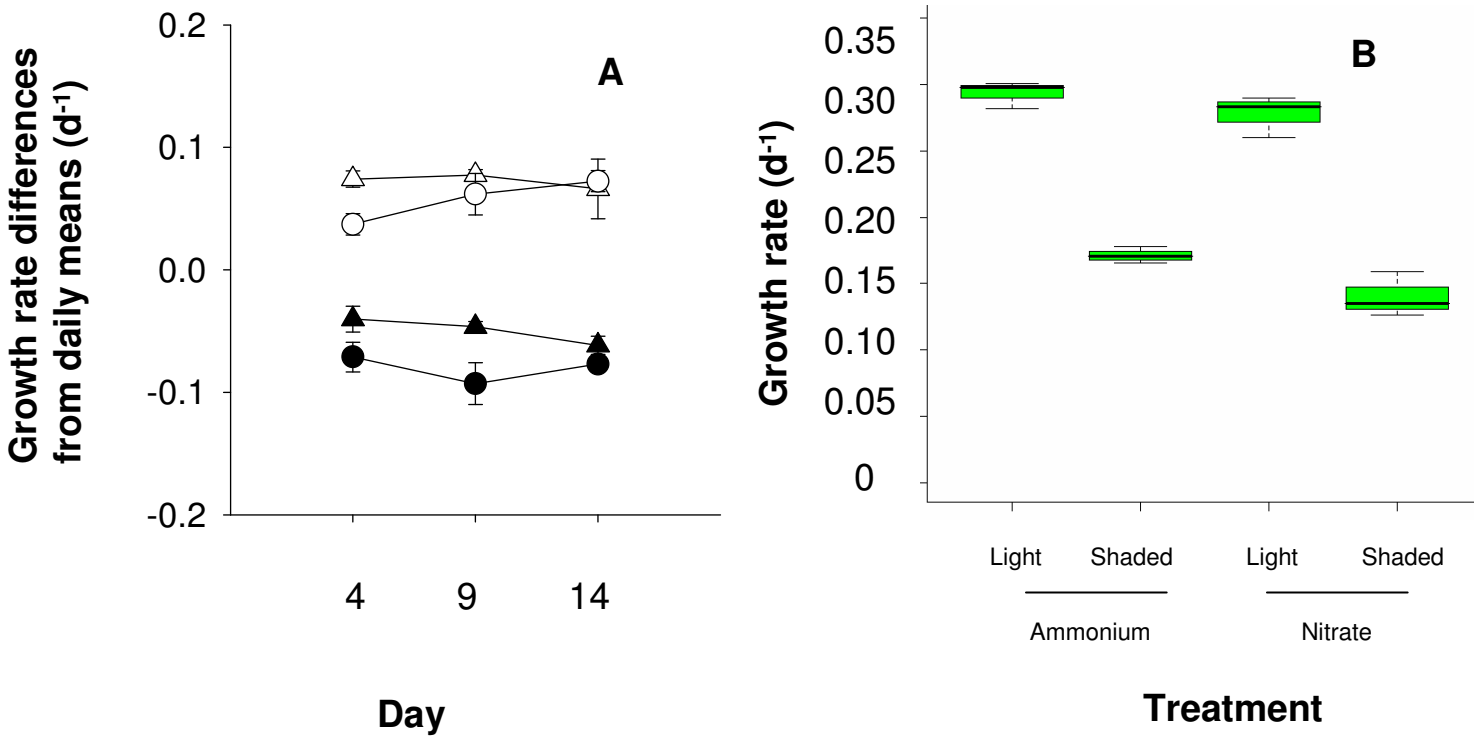
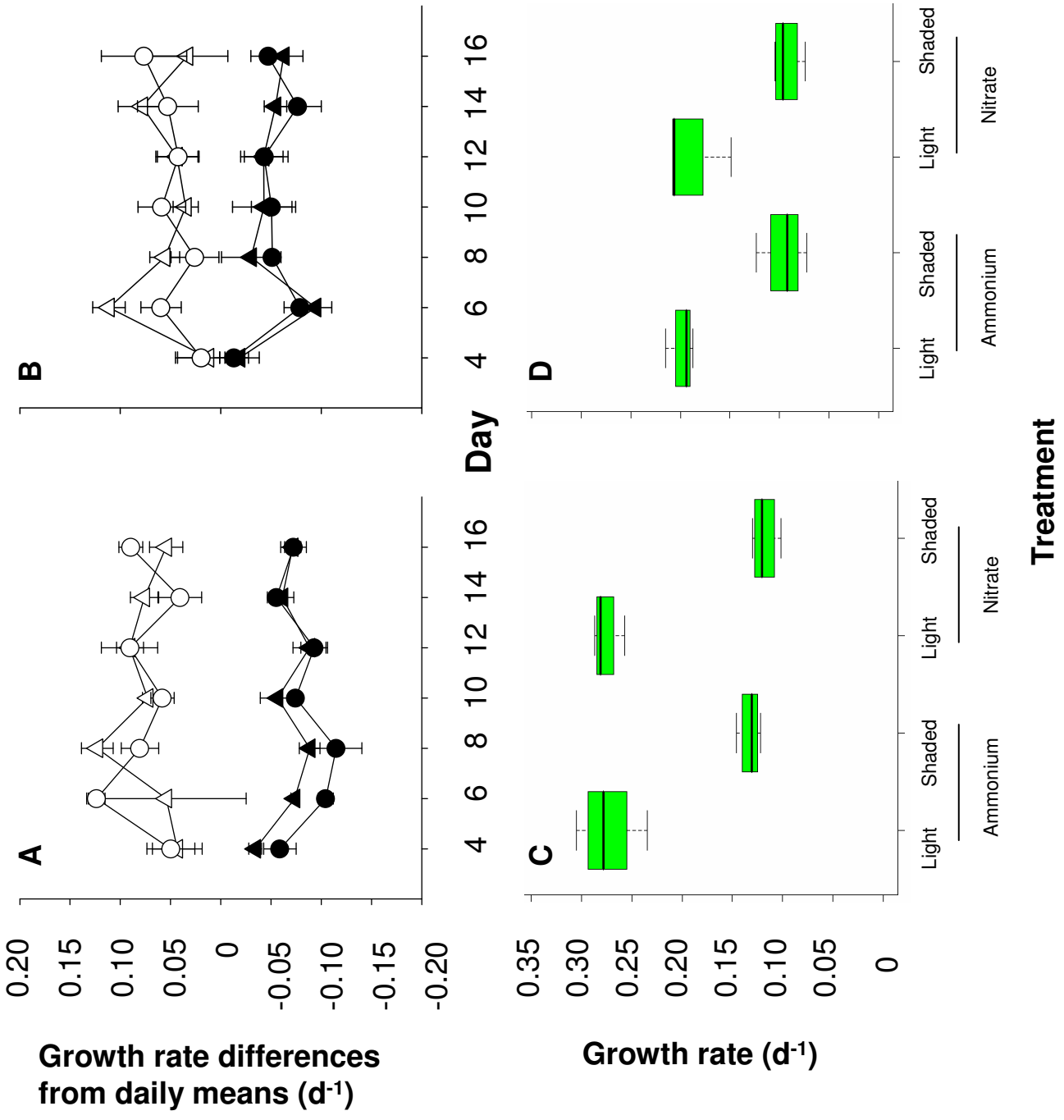


Figure 3.2. **A**. Experiment 1 - 2004/2005. Time series of residual growth rates of *Ulva sp.* after accounting for changes in growth rate due to changing ambient environmental conditions (e.g. sunlight). Figures displayed are treatment means of chamber growth rate – mean growth rate for all chambers during that period. Given are means +/- S.E. n = 3 for each point. Ammonium treated plants given as triangles, nitrate treated plants given as circles. Ambient light treated plants given as white symbols, reduced light treated plants given as black symbols.
B. Experiment 1. - 2004/2005. Time-averaged growth rates (+/- S.E.) for all treatments. n = 3 for each point Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment.

Figure 3.3
Plot **A**. Residuals of growth rate means for treatments for experiment 2. - 2005/2006. Ammonium treated plants are represented by triangles, nitrate treated plants are represented by circles. Ambient light treated plants have white symbols, reduced light treated plants have black symbols. Given are means +/- S.E.
Plot **B**. Residuals of growth rate means for treatments for experiment 3 - 2005/2006. Ammonium treated plants are represented by triangles, nitrate treated plants are represented by circles. Light treated plants have white symbols, reduced light treated plants have black symbols. Given are means +/- S.E.
Plot **C**. Mean growth rates for all treatments – Experiment 2. – 2005/2006
Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment
Plot **D**. Mean growth rates for all treatments – Experiment 3. – 2005/2006 Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment



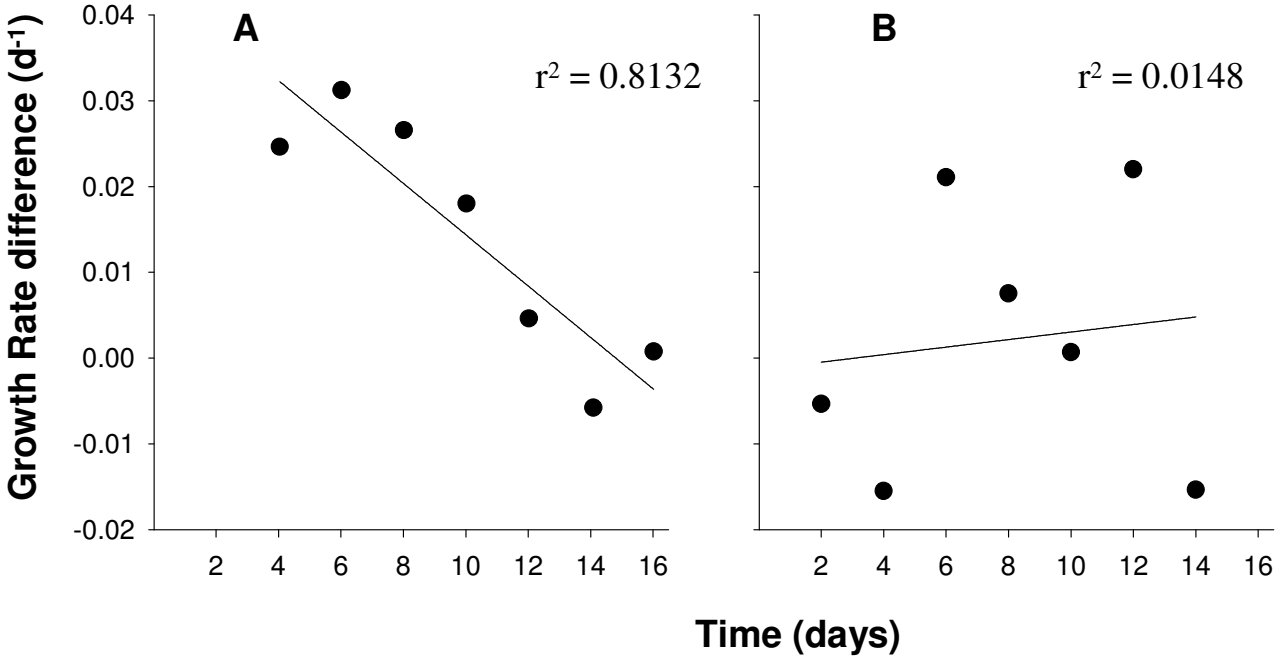


Figure 3.4.
Growth differences between nitrate and ammonium plants grown in shaded conditions in Experiment 2. - 2005/2006 (Plot **A**) and Experiment 3 (Nutrient source acclimatised tissue) 2005/2006 (Plot **B**). Each point represents differences in two-daily growth rates between the two treatments.

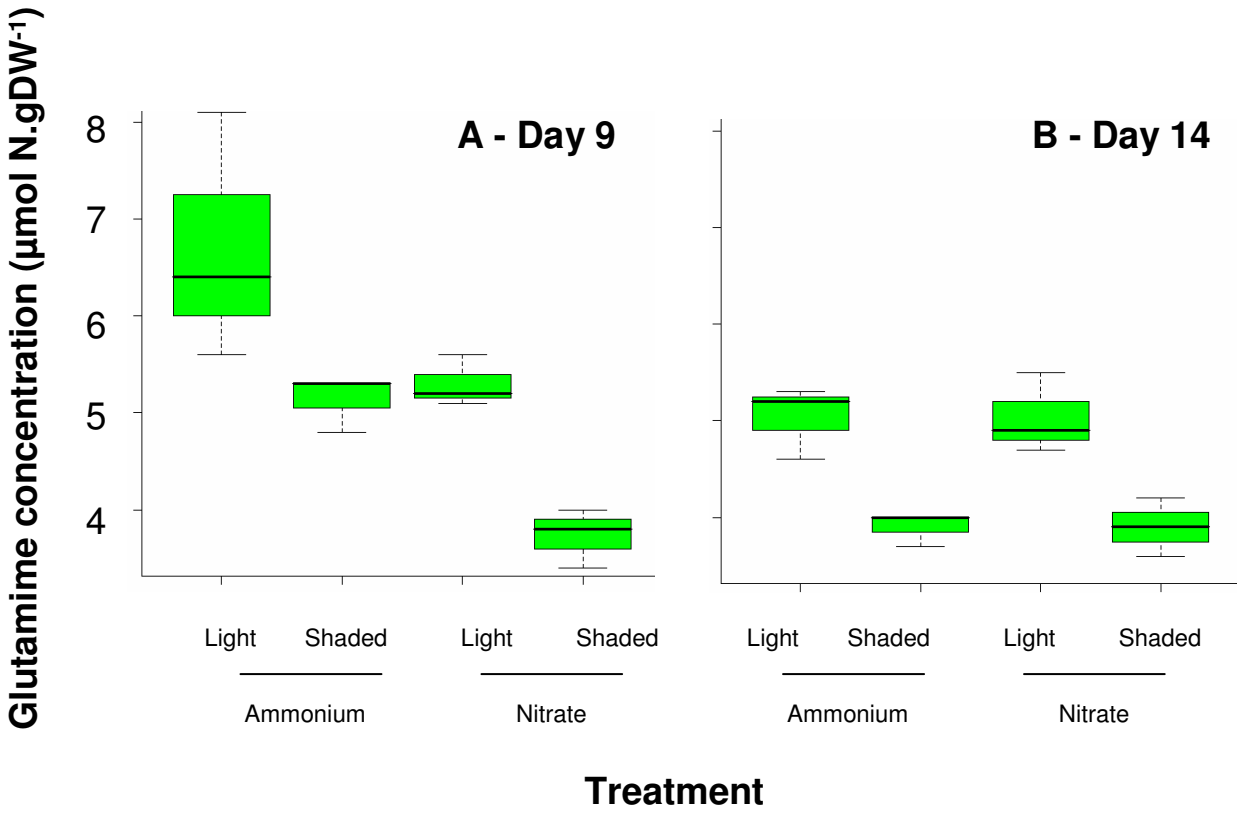


Figure 3.5 Plot **A**. Mean tissue glutamine levels for all treatments. Tissue samples taken on day 9 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment
Plot **B**. Mean tissue glutamine levels for all treatments. Tissue samples taken on day 14 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment

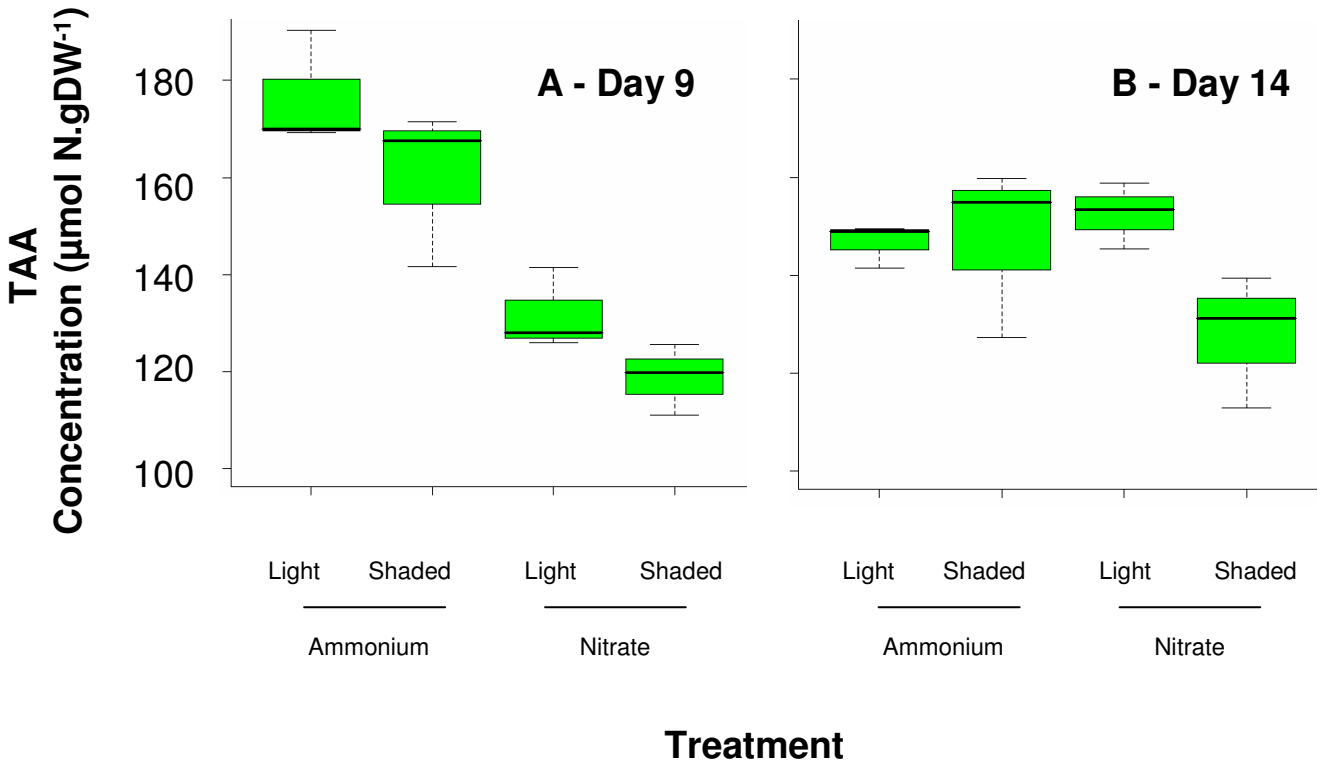


Figure 3.6.
Plot **A**. Mean tissue Total Free Amino Acid (FAA) levels for all treatments. Tissue samples taken on day 9 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment
Plot **B**. Mean tissue Total Free Amino Acid (FAA) levels for all treatments. Tissue samples taken on day 14 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment

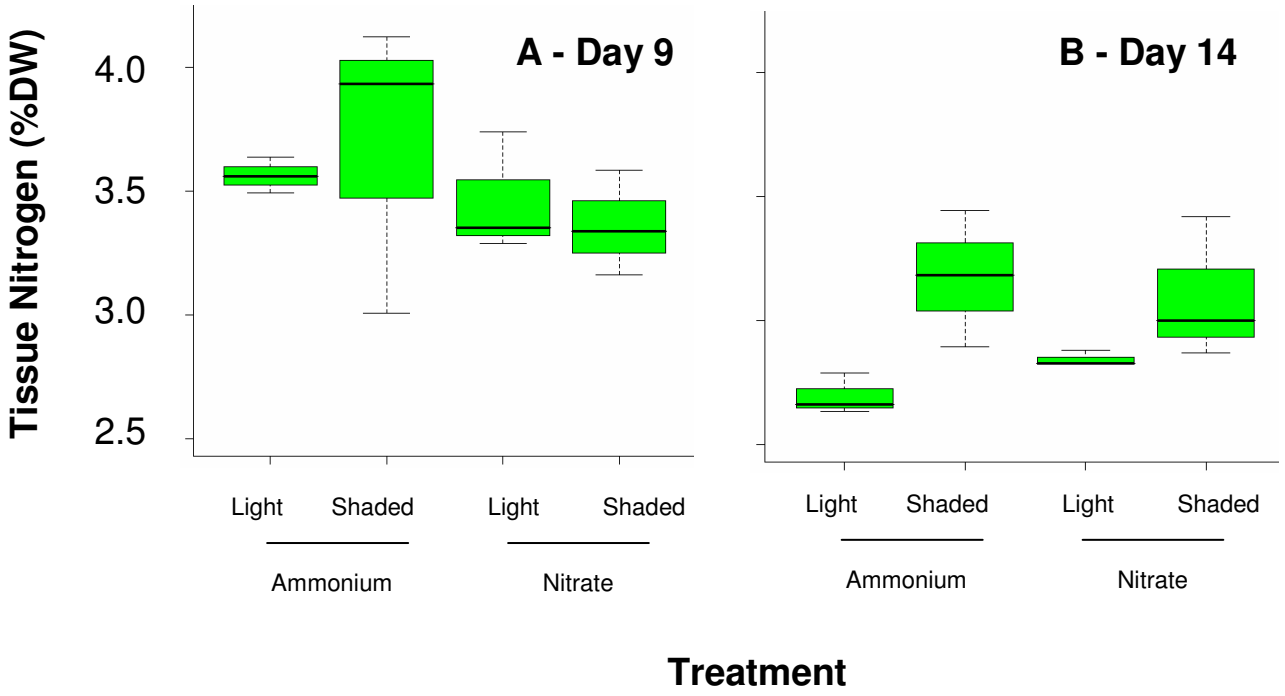


Figure 3.7 Plot **A**. Mean tissue nitrogen content for all treatments. Tissue samples taken on day 9 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment
Plot **B**. Mean tissue nitrogen content for all treatments. Tissue samples taken on day 14 of Experiment 1. – 2004/2005. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment

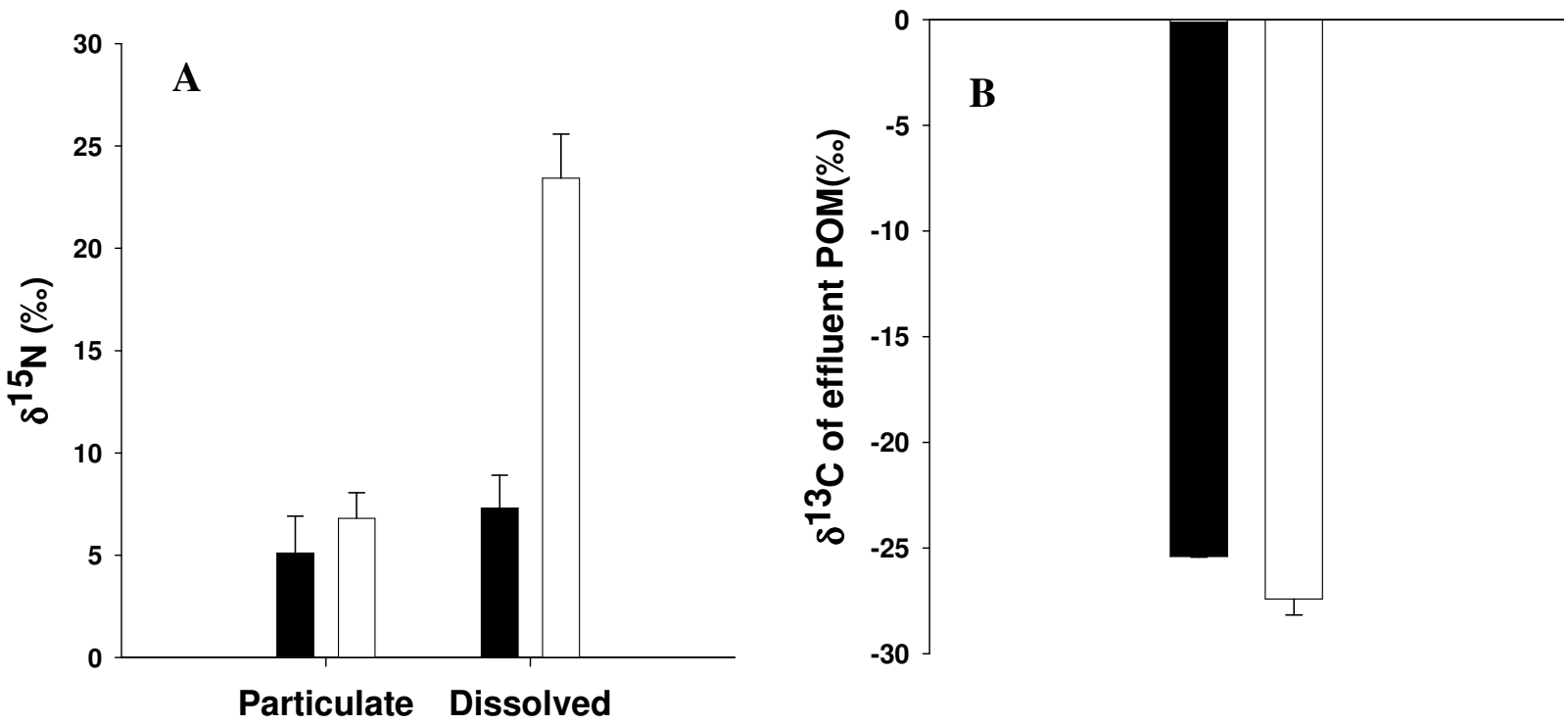


Figure 4.1.
Plot **A** - $\delta^{15}N$ levels in TBWWTP dissolved inorganic nitrogen (DIN) and sewage particulate organic matter (POM)
Plot **B** - $\delta^{13}C$ levels of sewage POM. Black bars give values for primary processed effluent, white bars give values for tertiary processed effluent. Error bars +/- 1 S.E.

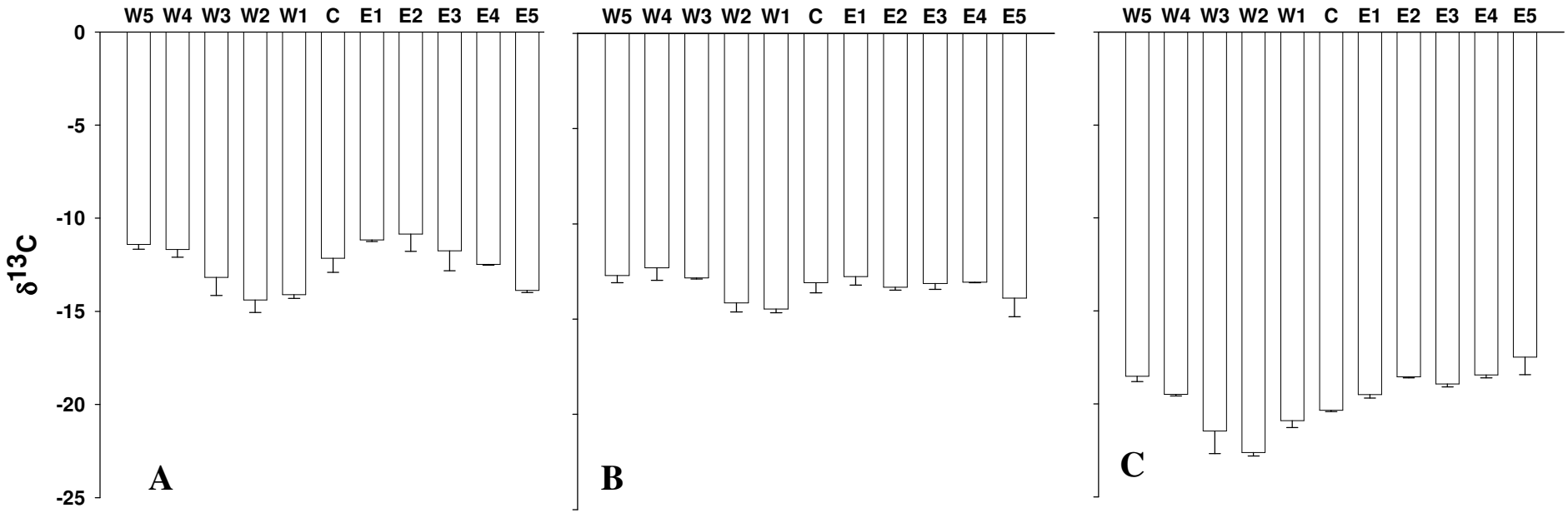


Figure 4.3. Time averaged $\delta^{13}C$ ratios in tissue of *C. maschalocarpum* **(A)**, *A longipes* **(B)** and *P. elongatus* **(C)** by site. Site marked C on the x-axis represents the sampling site at the outflow point to the TBWWTP, sites marked W1-W5 are at increasing distances to the west of the outflow, E1-E5 at increasing distances to the East of the outflow. n=3, Error bars +/- 1 S.E.

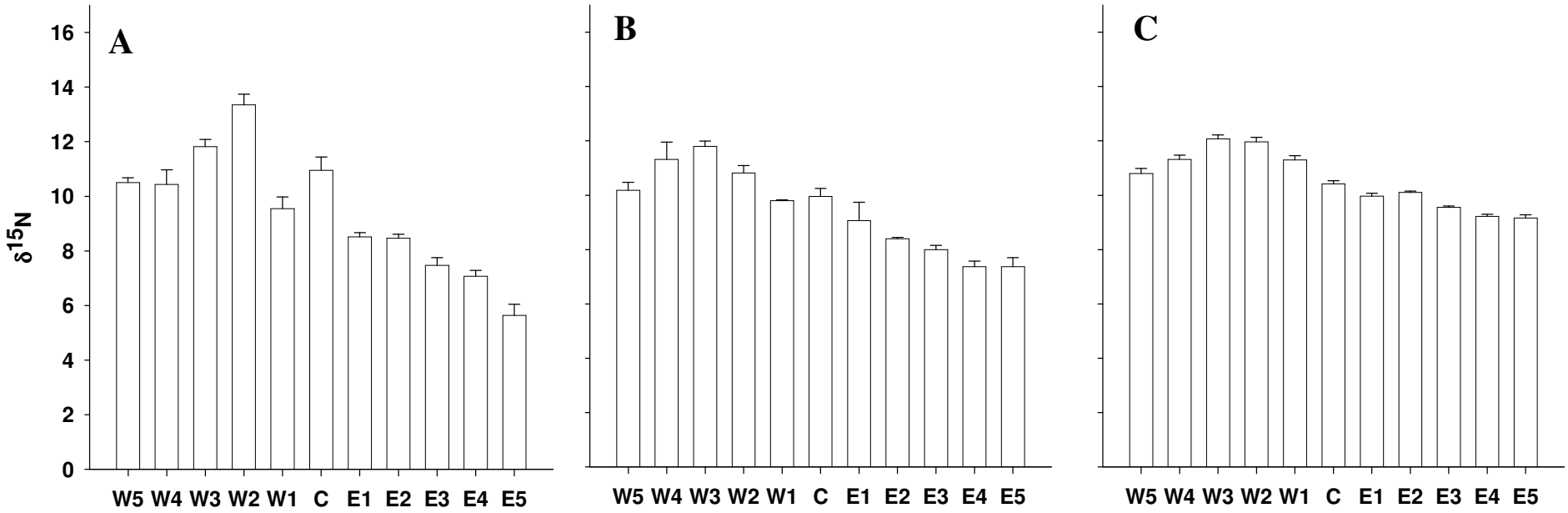


Figure 4.4. Time averaged tissue $\delta^{15}N$ values in *C. maschalocarpum* **(A)**, *A longipes* **(B)** and *P. elongatus* **(C)** by site. Site marked C on the x-axis represents the sampling site at the outflow point to the TBWWTP, sites marked W1-W5 are at increasing distances to the west of the outflow, E1-E5 at increasing distances to the East of the outflow. n=3, error bars +/- 1 S.E.

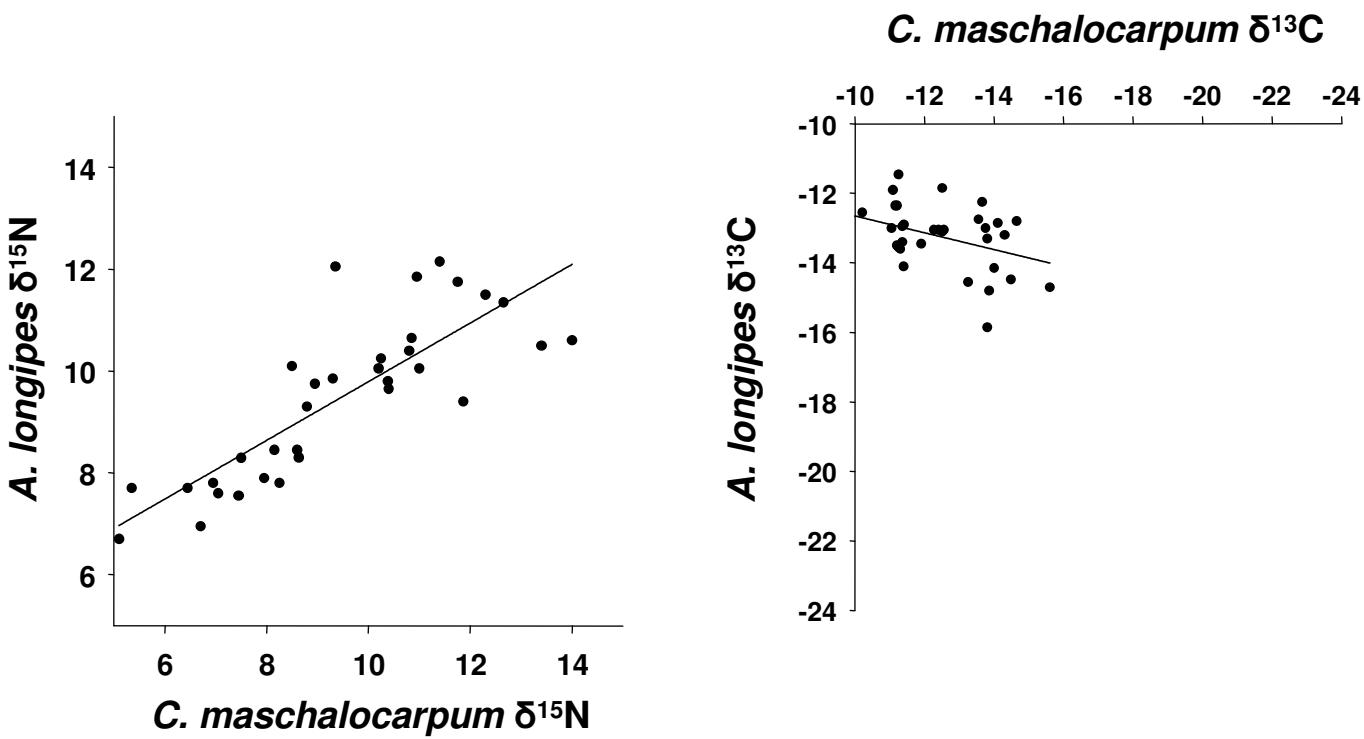


Figure 4.5. Regression plots of the relationship between tissue $\delta^{13}C$ and $\delta^{15}N$ values in individual *C. maschalocarpum* plants and those of the epifaunal grazer *A. longipes* collected from their surface.

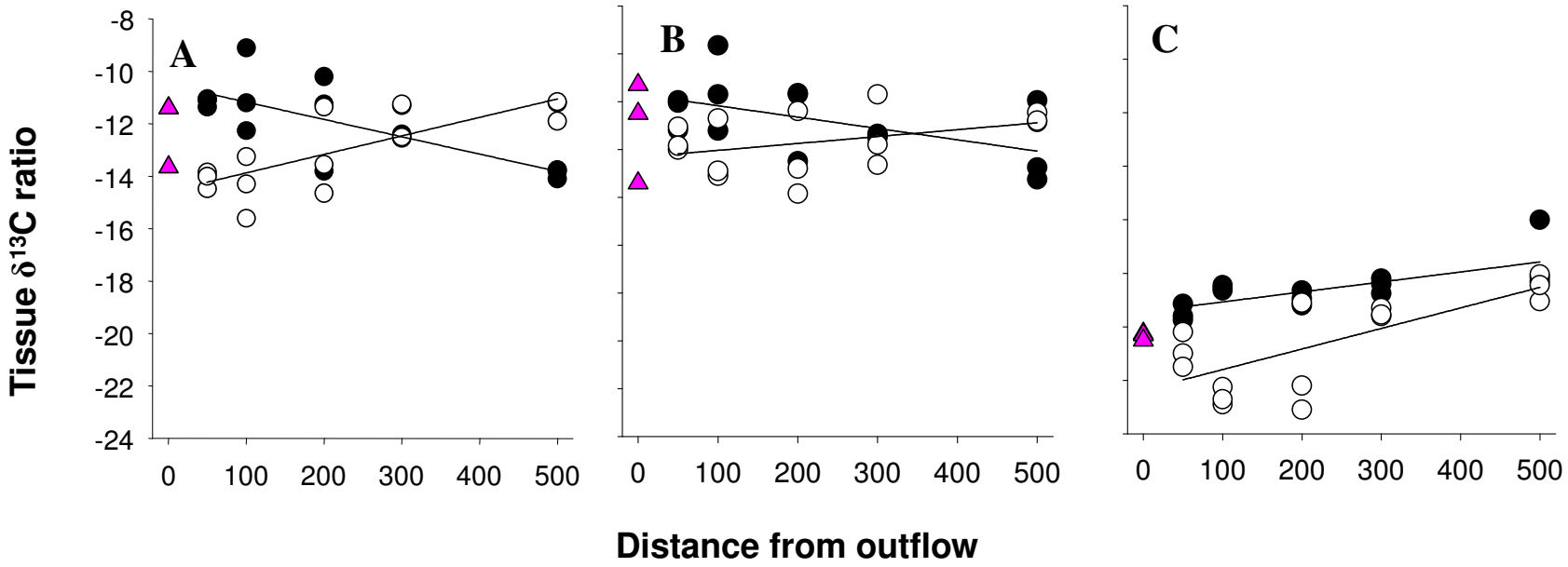


Figure 4.6. Tissue $\delta^{13}C$ ratios of *C. maschalocarpum* (plot **A**), *A. longipes* (Plot **B**), and *P. elongatus* (Plot **C**) at distance from the TBWWTP. Data points are marked with black circles (east sites), white circles (west sites), and pink triangles (centre site). Significant effects of distance or direction in ANCOVA analysis are highlighted with regression lines through separate East or West data sets. Slopes and intercepts of regression lines presented are based on separate regression analysis of 'surface' or '4m' data sets. Centre sites are not included in regression fits.

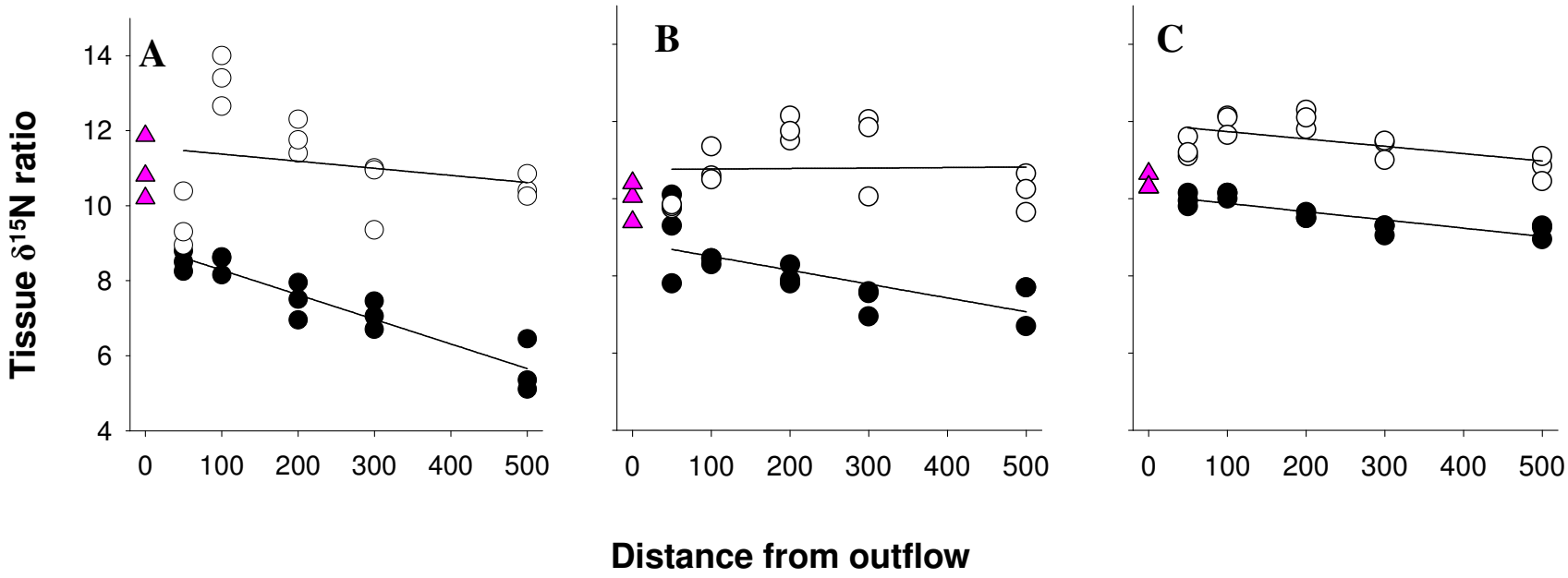


Figure 4.7. Tissue δ^{15}N ratios of *C. maschalocarpum* (plot **A**), *A. longipes* (Plot **B**), and *P. elongatus* (Plot **C**) at distance from the TTBWWTP. Data points are marked with black circles (east sites), white circles (west sites), and pink triangles (centre site). Significant effects of distance or direction in ANCOVA analysis are highlighted with regression lines through separate East or West data sets. Slopes and intercepts of regression lines presented are based on separate regression analysis of 'surface' or '4m' data sets. Centre sites are not included in regression fits.

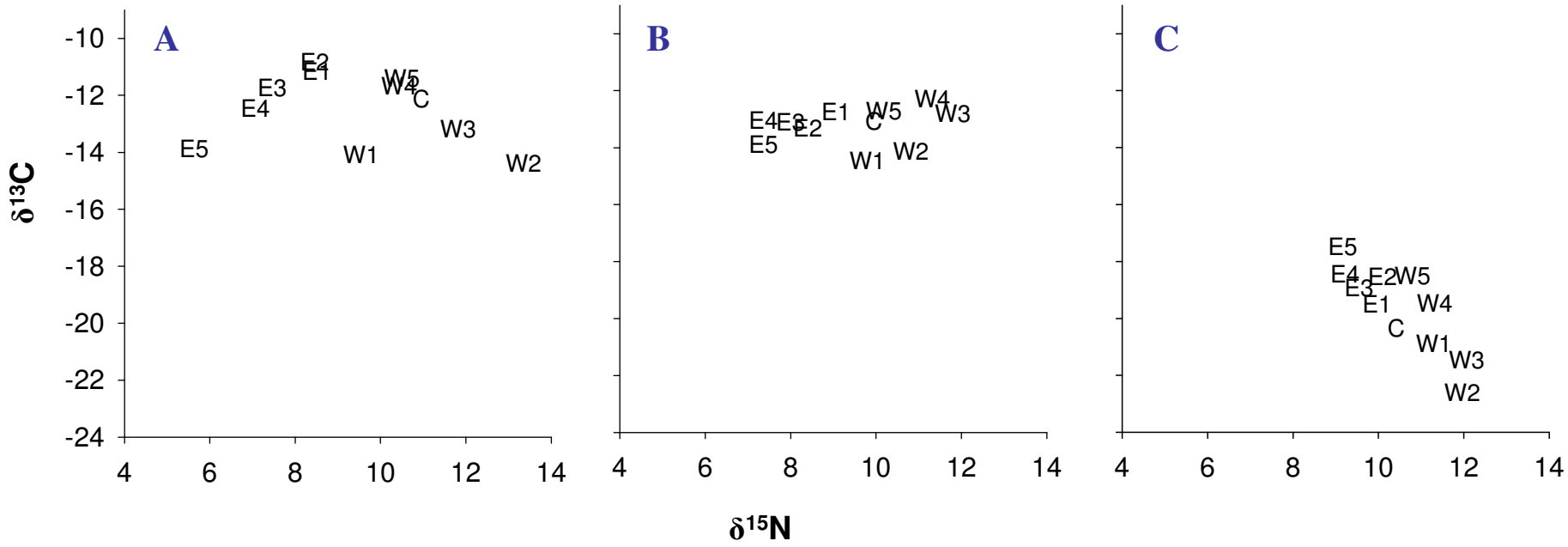


Figure 4.8. $\delta^{15}N$ vs $\delta^{13}C$ plots of *C. maschalocarpum* (Plot **A**), *A. longipes* (Plot **B**) and *P. elongatus* (Plot **C**) at 11 sites with varying sewage influence. Shown are time averaged values. Standard errors for time averaged means of $\delta^{13}C$ and $\delta^{15}N$ are shown in figures 5.3 and 5.4 respectively.

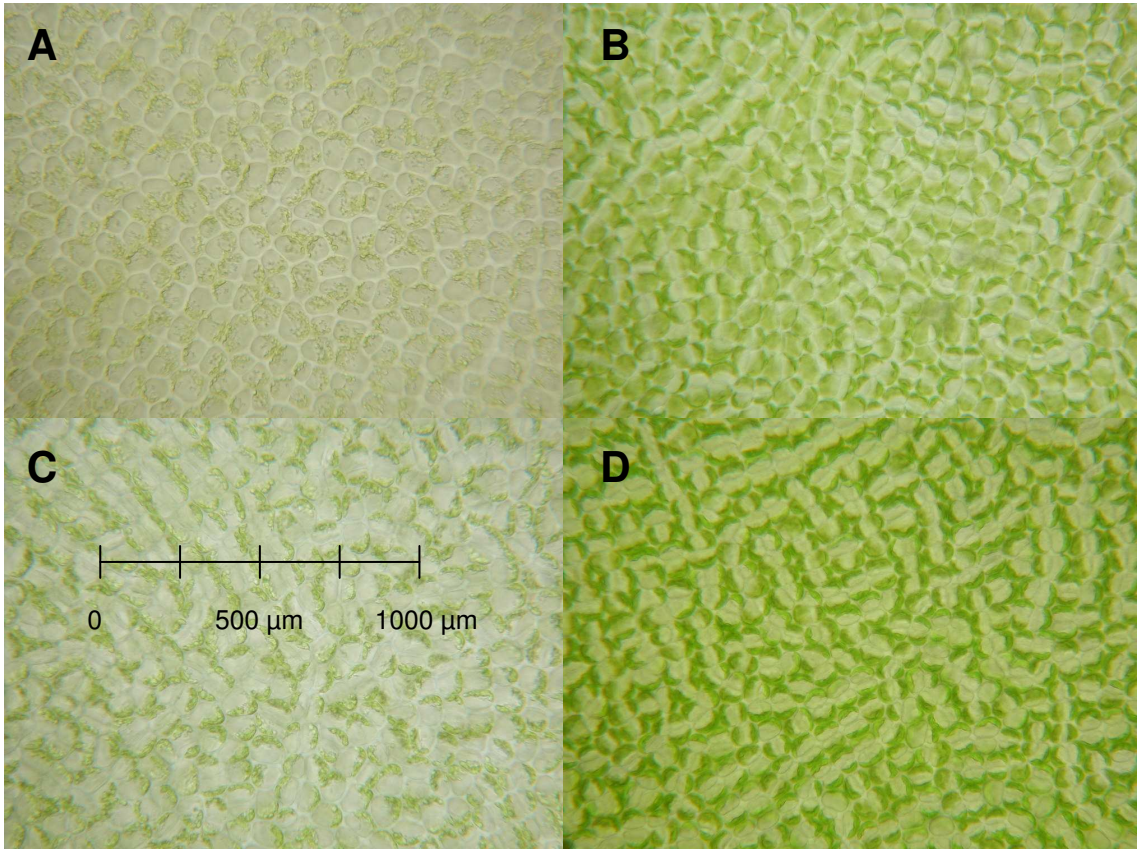


Figure 7.1 Magnification of *Ulva* sp. cells after treatment with the factorial regime of light and nutrient additions described in the experiment from chapter 2. Photos above correspond to (Photo **A** - ambient light/no nutrient addition), (Photo **B** - Ambient light, added 10µM ammonium and 1µM Phosphate) (Photo **C** - Shaded/no nutrient addition), and (Photo **D** - Shaded/ added 10µM ammonium and 1µM Phosphate).

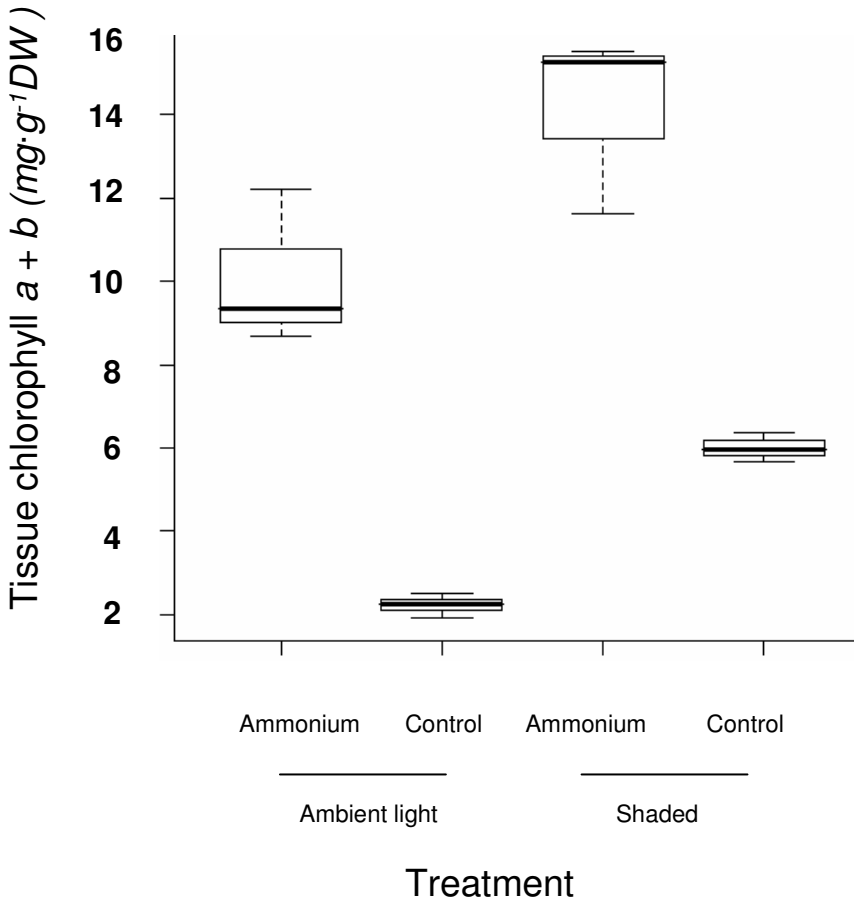


Figure 7.2. Effects of light and nutrient availability on the chlorophyll content of *Ulva* sp. tissue after 14 days of sustained growth. Whiskers on plot give data range, dark bars at the centre of boxes represent the median for each treatment, upper and lower limits of each box give the upper and lower quartiles for each treatment.

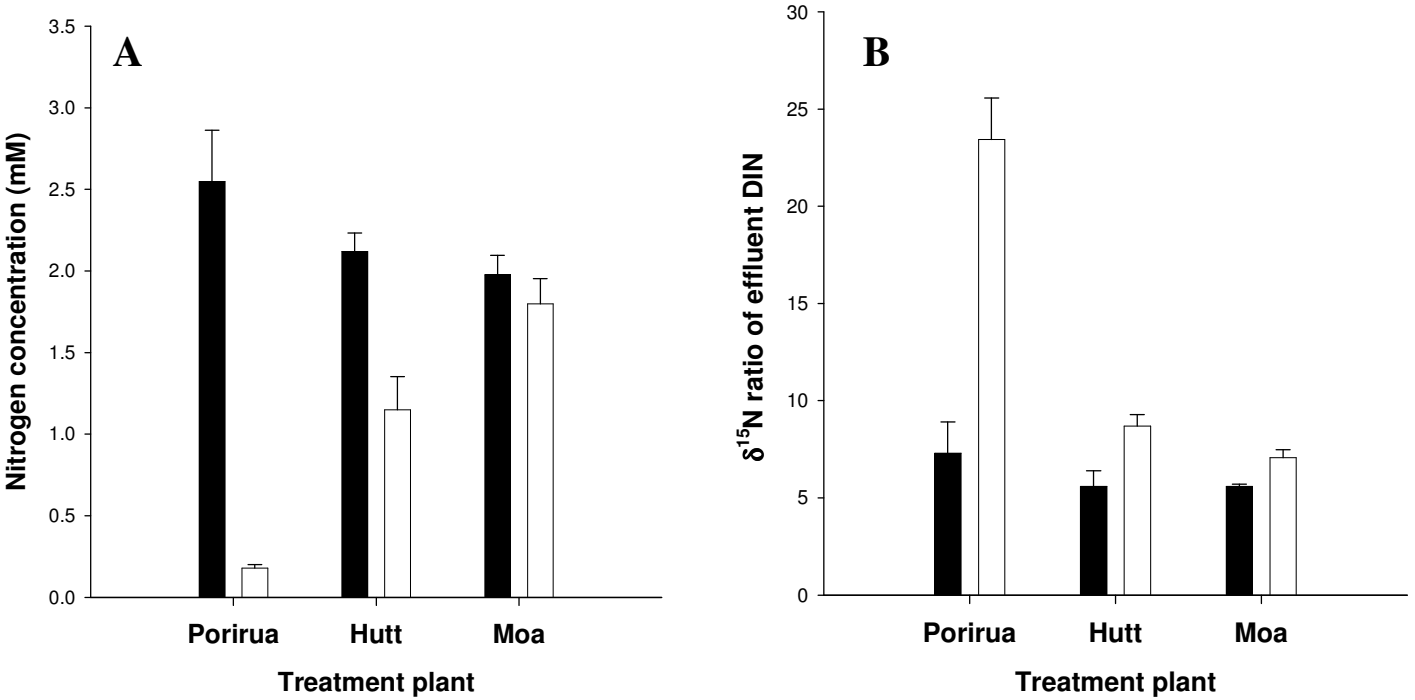


Figure 10.1. Dissolved inorganic nitrogen (DIN) concentration **(A)** and DIN $\delta^{15}N$ signature **(B)** in primary and tertiary processed effluent at three WWTP's in the Wellington region. Black bars give values for primary processed effluent, white bars give values for tertiary processed effluent. n =3, Error bars +/- 1 S.E.

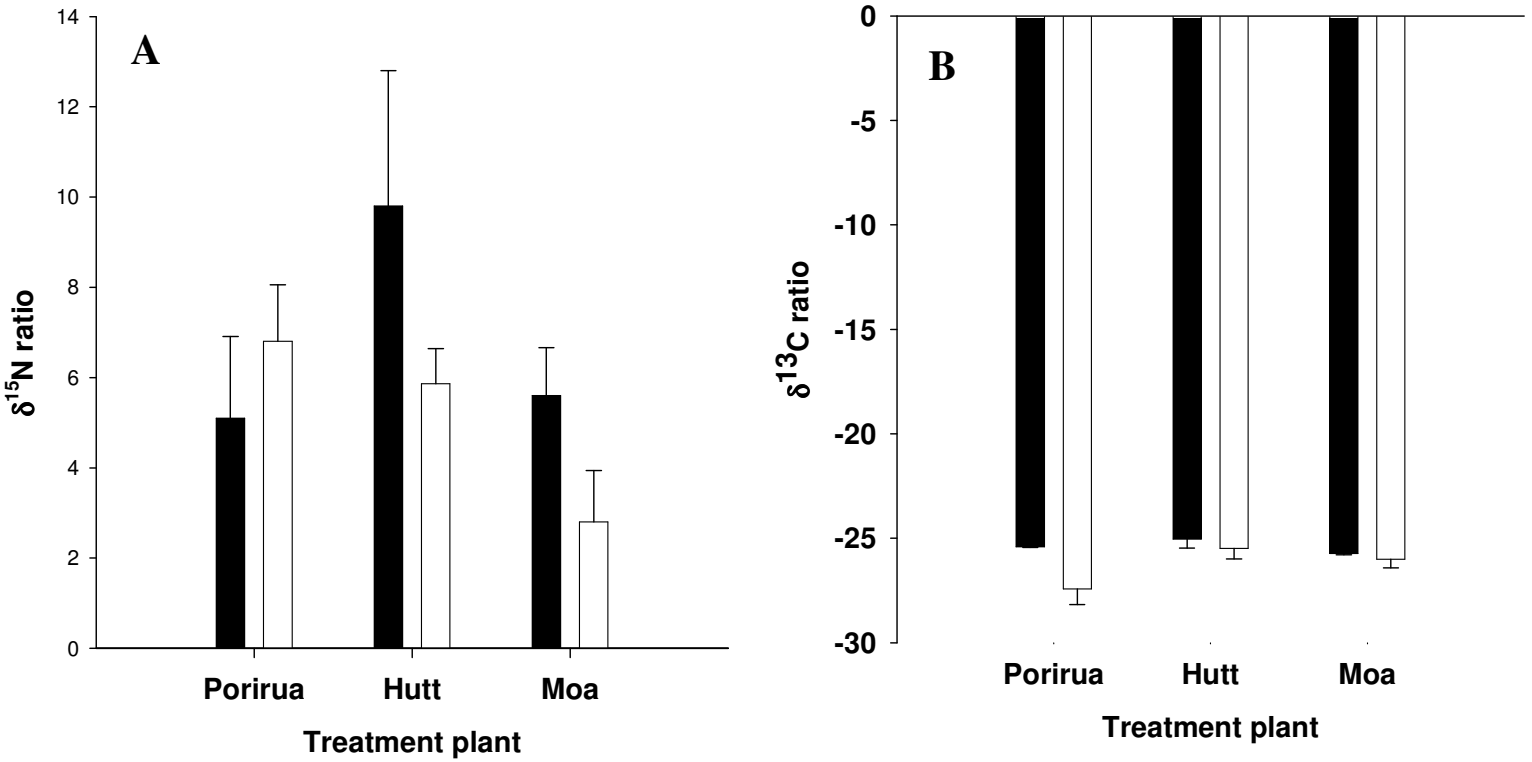


Figure 10.2. $\delta^{15}N$ (plot A) and $\delta^{13}C$ (plot B) ratios of the particulate fraction of effluent in primary (black bars) and tertiary (white bars) processed effluent from WWTPs in the Wellington region. n=3, Error bars +/- 1 S.E.