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# Chapter One
# General introduction

## 1 General patterns of nitrogen utilisation in macroalgae

### Background

Seaweeds, or marine macroalgae, are some of the most productive macrophytes on Earth (Mann *et al.*, 1980; DeBoer, 1981). In order to sustain this high productivity, macroalgae require inorganic carbon, water, light and various mineral ions for photosynthesis and growth (Lobban and Harrison, 1997). At least 21 elements are required to maintain the main metabolic processes in plants, although more than twice this number are present in (or closely associated with) seaweeds (DeBoer, 1981). Some of these elements are found in seawater in relative abundance, while others may only be found in low concentrations or in a form not readily available to the plant and, as such, are termed "limiting nutrients". According to Liebig's law of the minimum, when other factors such as light and temperature are more than adequate for growth, the nutrient available in the smallest quantity with respect to the requirements of the plant will limit the overall yield (DeBoer, 1981). In contrast, Blackman-type limitation is when the availability of a given nutrient limits the rate of photosynthesis, nutrient utilisation or growth. Of all the limiting nutrients, nitrogen (N) has probably received the most attention and in temperate coastal environments nitrogen availability may frequently limit the rate of growth and / or yield of seaweeds (Mann, 1972; Hanisak, 1983; Smith, 1984).

### Nitrogen and growth in seaweeds

In general, nutrient-poor regions are characterised by slow-growing species of macroalgae while fast-growing species are dominant in nutrient-rich waters (Pedersen and Borum, 1997). The relationship between growth and nutrient uptake is complicated by changes in nutrient supply that may vary on time-scales ranging from hours (e.g. tidal) to months (e.g. seasonal) (McGlathery *et al.*, 1996). In winter,

internal nitrogen reserves in slow-growing, perennial seaweeds can be two or three times higher than in summer (Chapman and Craigie, 1977; Hanisak, 1983; Hanisak and Samuel, 1983). As a result of this, growth and nutrient uptake may be uncoupled. A classic example of the relationship between the uncoupling of uptake of nitrogen and growth is the kelp, *Laminaria*. In the summer when seawater nitrate levels are low and photosynthesis is most active, carbon is stored, which can then be used for growth in the autumn when nitrate levels increase (Mann, 1982). These seaweeds store intracellular nitrate in winter, which can be used during periods of N-deficiency. In spring growth is dependent on stored nitrate (Mann, 1982).

In a study of the relationship between N-supply and growth, Pedersen & Borum (1997) conclude that fast-growing seaweeds, including *Ulva lactuca*, which are characterised by high SA : V ratio and high rates of uptake and growth, have a high nitrogen requirement. Most of this requirement is due to a high demand for nitrogen associated with their high growth rate and, to a lesser extent, because of their high tissue-N content. In addition, the ratio of maximum rates of nitrogen uptake to nitrogen requirement is lower in fast-growing species (Pedersen and Borum, 1997). This high demand for nitrogen, coupled with the requirement for a higher nitrogen concentration to saturate growth, explains why fast-growing macroalgae can dominate in areas with excess nutrient supplies.

**Sources of nitrogen in seawater**

Elemental nitrogen is in abundant supply in both the air and seawater, but there is no evidence that macroalgae are able to utilise it directly (DeBoer, 1981). In contrast, sources of inorganic nitrogen in seawater are often present in low concentrations. The majority of continental shelf edges of the world's oceans do not have strong, persistent upwelling (Sharp, 1983). However, the occurrence of upwelling in coastal areas can dramatically increase inorganic nitrogen levels, with subsurface nitrate levels approaching 30 μM (Hanisak, 1983). In the majority of coastal zones nitrate typically ranges in concentrations of 0-30 μM (Sharp, 1983). Ammonium (in the context of this study, the sum of $NH_3$ and $NH_4^+$) concentrations in coastal areas are usually below 3 μM, although inputs from terrestrial sources, such as sewage, may increase values to 20-25 μM or more (Sharp, 1983). Nitrite concentrations are almost always low and rarely exceed 5 % of the nitrate level, while ammonium

concentrations generally comprise less that 20 % of total inorganic nitrogen in seawater (Sharp, 1983).

Macroalgae, and algae in general, utilise nitrogen from a variety of sources, which along with the other nutrients they require is usually taken up directly from the water that surrounds them (Lobban *et al.*, 1985). Macroalgae can utilise organic sources of nitrogen, such as urea (DeBoer, 1981), and in some cases, amino acids, pyrimidines and purines (Hanisak, 1983). It has also been shown that some species of macroalgae can utilise ammonium released by invertebrates such as barnacles (Williamson and Rees, 1994) and bryozoans (Hurd *et al.*, 1994a) and other epifauna which live in close proximity (Taylor and Rees, 1998). Overall, probably the most important nitrogen sources available to macroalgae are nitrate and ammonium (Hanisak, 1983; Lobban and Harrison, 1997).

**Ammonium and nitrate uptake, and utilisation in macroalgae**

Ammonium is often taken up at higher rates than nitrate (D'Elia and DeBoer, 1978; Haines and Wheeler, 1978; Hanisak and Harlin, 1978; Topinka, 1978; Morgan and Simpson, 1981; Wallentinus, 1984; Thomas and Harrison, 1985; Rees, 2003), despite the fact that nitrate is often the more abundant source of inorganic nitrogen in coastal waters (DeBoer, 1981). Moreover, it has been shown that the presence of ammonium in the medium can inhibit nitrate uptake in many seaweed species (D'Elia and DeBoer, 1978; Haines and Wheeler, 1978; Hanisak, 1983; Thomas and Harrison, 1987) and, in some cases, uptake of nitrate may be inhibited by as much as 50% (Thomas and Harrison, 1987). Similarly, the presence of nitrate has been shown to inhibit the uptake of nitrite (Hanisak and Samuel, 1983). In some instances, nitrate may be taken up at an equivalent (Probyn and Chapman, 1983; Thomas *et al.*, 1987a) or even higher rate than ammonium, in N-deficient plants (Thomas *et al.*, 1987b).

Utilisation of ammonium in preference to nitrate is theoretically energetically cheaper for algae (Syrett, 1981; Turpin *et al.*, 1991; Vergara *et al.*, 1995). The reduction of, firstly nitrate to nitrite (catalysed by nitrate reductase with NADPH or NADH as the electron donor), and then nitrite to ammonium (catalysed by nitrite reductase in the chloroplasts with reduced ferredoxin as the electron donor), represents a higher energy requirement than assimilation of ammonium (Syrett, 1981; Turpin *et al.*, 1991;

Vergara *et al.*, 1995). Under conditions of N-starvation preferential uptake of ammonium may not occur (Thomas and Harrison, 1985; Thomas and Harrison, 1987; Corzo and Niell, 1992), and it is probable that the requirement for N, when it is in limiting supply, outweighs the energetic advantage of ammonium utilisation in algae (Syrett, 1981). In support of these suggestions it has been shown that the efficiency of ammonium uptake is commonly greater than that for nitrate across a range of macroalgae (Rees, 2003).

**Factors affecting uptake and utilisation of nitrogen in macroalgae**

Light is of fundamental importance to the metabolism of ammonium by algae (Turpin and Harrison, 1978; Syrett, 1981; Lobban and Harrison, 1997) for four main reasons, because it:

- provides energy (ATP) for active transport and metabolism.
- provides charged ions that establish Donnan potentials (a requirement of the Donnan exchange system in seaweeds and multicellular plants).
- through photosynthesis (indirectly), provides carbon skeletons for ammonium assimilation and amino acid biosynthesis.
- increases growth, thereby increasing the demand for nutrients.

The relationship between irradiance and uptake of nitrate is described by a rectangular hyperbola (Wheeler, 1982). In contrast, ammonium uptake in seaweeds is largely independent of irradiance (Topinka, 1978; Wheeler, 1982; Duke *et al.*, 1989b; Coutinho and Zingmark, 1993), but can also show diel periodicity (D'Elia and DeBoer, 1978; Harrison *et al.*, 1986). Light quality (i.e. the wavelength of incident irradiance) is another important factor in the uptake of nitrogen by macroalgae (Corzo and Niell, 1992).

Temperature has a substantial effect on the active uptake of ammonium, typically exhibiting a $Q_{10}$ factor of 2 (i.e. for every 10 $^{o}$C rise in temperature there is a doubling of the rate of uptake), but for passive diffusion the effect is minimal ($Q_{10}$ = 1.0-1.2) (Lobban and Harrison, 1997). Positive correlation between ammonium uptake and temperature has been demonstrated in seaweeds (Hanisak and Harlin, 1978; Topinka, 1978; Amat and Braud, 1990), but temperature has a greater effect on growth than on

uptake of nitrate and ammonium (Duke *et al.*, 1989b). Moreover, temperature has little effect on ammonium uptake by *Gracilaria verrucosa* (Ventura and Harlin, 1976). This may be either because seaweeds can compensate for lower enzyme activities at lower temperatures by increasing the enzyme concentrations (Duke *et al.*, 1989b), or because uptake is passive. However, it appears that the effect of temperature on ion uptake in seaweeds is both ion and species specific (Lohaus and Heldt, 1997).

Water motion and turbulence is thought to be an important factor in the uptake of ammonium, and inorganic nutrients in general (Parker, 1981; Wheeler, 1982; Hurd *et al.*, 1994b; Hurd *et al.*, 1996; Larned and Atkinson, 1997; Barr *et al.*, In preparation). Moreover, growth of *Ulva* may become limited in still water, because uptake of ammonium is inhibited due to the development of thick diffusion boundary layers around the thallus (Parker, 1981; Barr *et al.*, In preparation). Desiccation can also affect ammonium uptake directly since seawater, and therefore dissolved nutrients, are removed from the thallus surface. Ultimately, this is reflected in the N-status of the seaweed (Thomas *et al.*, 1987b). As a consequence there is an increased potential (or demand) for uptake of ammonium and nitrate upon re-supply of water in intertidal seaweeds (Thomas *et al.*, 1987a; Thomas *et al.*, 1987b; Thomas and Harrison, 1987).

The relationship between morphology and ability to take up nutrients from seawater has been investigated in different species of macroalgae (Rosenberg and Ramus, 1984; Hein *et al.*, 1995; Taylor *et al.*, 1998; Taylor *et al.*, 1999). Morphology is generally described by the surface area : volume ratio (SA : V) and there is a strong, positive relationship between SA : V and rates of ammonium uptake in seaweeds (Rosenberg and Ramus, 1984; Hein *et al.*, 1995; Taylor *et al.*, 1998; Taylor *et al.*, 1999). The development of hyaline hairs on the thalli of some seaweeds is considered to be a morphological adaptation that increases SA : V and enhances rates of nitrogen uptake (DeBoer and Whoriskey, 1983). It has been suggested that particular morphologies allow some seaweeds to exploit diel or tidal nutrient pulses (Duke *et al.*, 1987), and seaweeds that have high SA : V have higher rates of photosynthesis (Markager and Sand-Jensen, 1994; Taylor *et al.*, 1999), growth rates (Nielsen and Sand-Jensen, 1990) and rates of ammonium uptake and assimilation (Rosenberg and Ramus, 1984; Hein *et al.*, 1995; Taylor *et al.*, 1998; Taylor *et al.*, 1999). However,

macroalgae with high SA : V ratios may be limited in their ability to store nitrogen compared with those which have low SA : V ratios, such as *Gracilaria* and *Codium* (Rosenberg and Ramus, 1984; Duke *et al.*, 1987).

Seasonal (and biogeographic) patterns in rates of ammonium and nitrate uptake (Gagne *et al.*, 1982), and growth (Chapman and Craigie, 1977; Chapman and Lindley, 1980; Gagne *et al.*, 1982) have often been described in seaweeds. Generally these relate to the physical factors already described: seasonal changes in incident light levels and quality (related to turbidity and depth), temperature, desiccation and wave energy. Seasonal changes in nutrient availability in seawater, as are evident in spring blooms of phytoplankton, have also been shown to affect growth patterns of macroalgae (Gagne *et al.*, 1982; Probyn and Chapman, 1983). For example, uptake of ammonium (and nitrate and urea) may become enhanced (reflected in lower $K_m$ values for uptake) over the summer months in some seaweeds (Probyn and Chapman, 1983). Moreover, ammonium and nitrate uptake may become uncoupled from growth in many seaweeds during periods of low N-availability (Chapman and Craigie, 1977; Topinka, 1978; Gagne *et al.*, 1982; Probyn and Chapman, 1983; Pedersen and Borum, 1997).

**Nitrogen assimilation and incorporation in macroalgae**

Uptake of inorganic nitrogen is followed by assimilation, which is defined as the enzymatic conversion of ammonium into amino acids. Generally much more is known about uptake of ammonium in macroalgae compared with that of its assimilation. Ammonium is an important source of inorganic nitrogen for macroalgae and marine algae in general (D'Elia and DeBoer, 1978; Raven *et al.*, 1993). It has also been suggested that there should be an energetic advantage for macroalgae to preferentially utilise ammonium since it can be assimilated directly while other nitrogen sources (e.g. nitrate and nitrite) have to be converted to ammonium before they can be assimilated (Syrett, 1989).

The metabolism of nitrogen by marine algae is broadly described by five processes and is summarised below (Figure 1.1):

1) uptake (by passive or facilitated diffusion, or active transport) of nitrogen across the cell membrane.
2) cellular storage in acidic compartments, primarily the vacuole.
3) assimilation of ammonium through enzymatic conversion to small organic metabolites (e.g. amino acids, purines, pyrimidines), which will be counteracted by amino acid catabolism (e.g. photorespiration).
4) incorporation of small organic metabolites into macromolecules (e.g. protein, nucleic acids), which will be counteracted by catabolism (e.g. protein turnover).
5) utilisation of macromolecules for differentiation or growth.

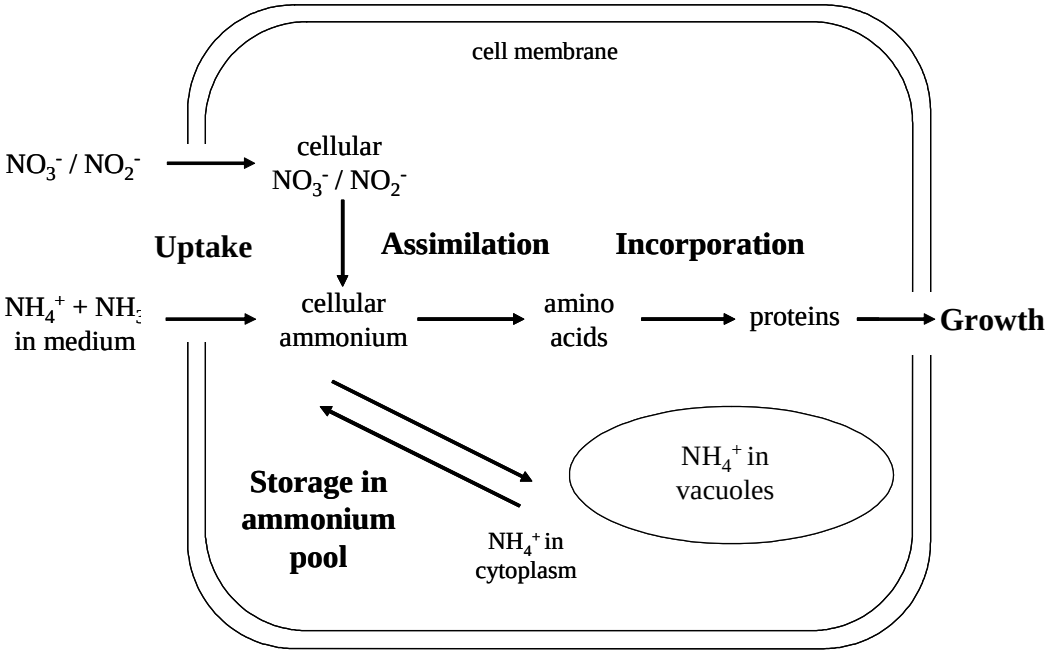



**Figure 1.1.** Schematic representation of nitrogen utilisation in seaweeds. Modified from Wheeler (1983) and Syrett (1981).

The primary route for ammonium assimilation in higher plants and microalgae is the glutamine synthetase / glutamine : 2-oxoglutarate aminotransferase (GS / GOGAT) pathway (Lea and Miflin, 1974; Syrett, 1981; Davison and Stewart, 1984; Ahmad and Hellebust, 1988a; Zehr and Falkowski, 1988; Syrett, 1989; Corzo and Niell, 1992). It is now also generally agreed that this is the primary pathway for ammonium assimilation in macroalgae (Haxen and Lewis, 1981; Davison and Stewart, 1984; Barr *et al.*, 2004). Evidence for GS / GOGAT being the primary ammonium assimilation

pathway in microalgae (Syrett, 1981) and macroalgae (Haxen and Lewis, 1981) is the high affinity of glutamine synthetase (GS) for ammonium (in comparison with other potential assimilatory enzymes, such as glutamate dehydrogenase) and the inhibition of ammonium assimilation by methionine sulfoximine (MSX), an inhibitor of GS (Barr *et al.*, 2004). The assimilation of ammonium into glutamate via the GS / GOGAT pathway is of major significance because it represents the junction from which other pathways involved in N-metabolism then diverge (Flynn *et al.*, 1994). In addition, because of the requirement for 2-oxoglutarate, GS / GOGAT also arguably represents the most important point of interaction between photosynthesis and N-metabolism (Syrett, 1981; Syrett, 1989; Turpin *et al.*, 1991).

In the reaction catalysed by GS, a molecule of ammonium is combined with a molecule of glutamate to form glutamine, with energy for the reaction provided by the hydrolysis of ATP (Hames *et al.*, 1997). In the next reaction, catalysed by GOGAT, glutamate is formed from the reductive amination of 2-oxoglutarate (Syrett, 1981; Syrett, 1989; Turpin *et al.*, 1991). GOGAT yields two molecules of glutamate for every molecule of glutamate used in the GS reaction (Figure 1.2). Glutamate can be converted into other amino acids, including aspartate via aspartate aminotransferase (AspAT), while glutamine and aspartate can be converted into asparagine via

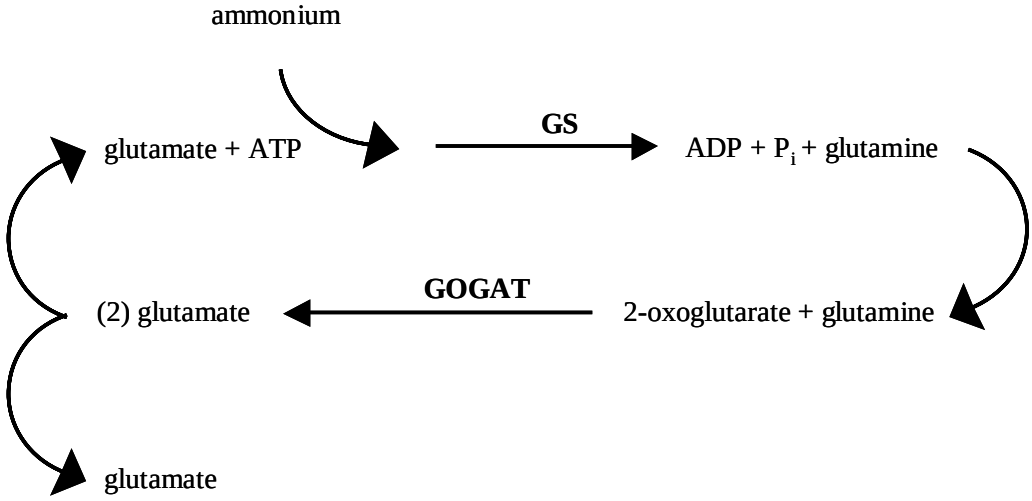



**Figure 1.2.** GS / GOGAT pathway of ammonium assimilation in algae. Modified from Syrett (1981).

asparagine synthetase (AS) (Oliveira *et al.*, 1997). In addition, AspAT and AS are considered to represent a second tier control of nitrogen assimilation (Vance, 1998).

**Nitrogen metabolism and free protein amino acids in macroalgae**

Other than the fundamental role of glutamine and glutamate in ammonium assimilation very little is known about the role and composition of other amino acids in macroalgae (Barr *et al.*, 2004). However, there can be little doubt that they play a vital role in nitrogen metabolism and are, along with amides, the first stable products of inorganic nitrogen assimilation (Oaks, 1994) as well as the building blocks for proteins. In higher plants it has been suggested that as free amino-acid concentration is largely dependent on N-status, changes in their cytoplasmic concentration may be involved in the regulation of plant growth and nitrogen uptake (Barneix and Causin, 1996). There is also considerable evidence that glutamine has a central role in regulating N-metabolism in plants (Oliveira *et al.*, 1997; Galvéz *et al.*, 1999; Oliveira and Coruzzi, 1999). For example, when supplied with external concentrations of glutamine and asparagine, ammonium uptake is reduced in wheat (Rodgers and Barneix, 1993). Similarly in maize roots, uptake of nitrogen decreases with increased accumulation of glutamine and asparagine (Lee *et al.*, 1992). Further clues to regulation of nitrogen metabolism come from various studies in microorganisms where it has commonly been shown that expression of GS genes is repressed by high levels of glutamine (Watanabe *et al.*, 1997). However, in studies of radish the ratio of glutamine : glutamate, as opposed to glutamine alone, was implicated as having a role in the expression of the gene for cytosolic glutamine synthetase (Watanabe *et al.*, 1997).

Glutamine and asparagine have been shown to accumulate to significant levels in *Ulva intestinalis* during short-term ammonium enrichment (Barr and Rees, 2003; Taylor *et al.*, 2006). Furthermore, the sequential increase in both these amino acids (i.e. first glutamine followed by asparagine), after provision of inorganic nitrogen, has been demonstrated (Taylor *et al.*, 2006). The accumulation in levels of asparagine after nitrogen enrichment might be expected since it is derived from the conversion of glutamine via AS. However, the actual role of asparagine in macroalgae is unknown although it has been suggested that asparagine synthesis may be a stress response to high ammonium in aquatic angiosperms (Smolders *et al.*, 2000) and to thermal stress

in *Ulva pertusa* (Kakinuma *et al.*, 2001). Asparagine is also more soluble and less reactive than glutamine (Sieciechowicz *et al.*, 1988) and thus may be suited to a storage role in at least some algae, such as *Ulva*.

Although not directly associated with nitrogen metabolism, proline has a significant role in the physiological responses of plants to environmental stressors. Accumulation of proline (an *alpha*-imino acid) is known to occur as a response to water deficits, salinity stress and temperature stress in higher plants (Matysik *et al.*, 2002) and algae (Kakinuma *et al.*, 2006). Proline accumulation in algae has also been shown to be a stress response to high levels of heavy metal contamination (Sharma and Dietz, 2006). In the study of Kakinuma et al (2006) proline accumulation in *Ulva* was shown to occur with high temperature stress. In higher plants, however, increases in proline are also associated with cold acclimation in winter (Dörffling *et al.*, 1997).

**Free amino acids as N-indices of nitrogen availability in macroalgae**

It is widely acknowledged that macroalgae accumulate nitrogen that is in excess of requirements for steady-state growth rate (Chapman and Craigie, 1977; Hanisak, 1979; Hanisak, 1983; Duke *et al.*, 1989b; Fujita *et al.*, 1989; Lavery and McComb, 1991). Moreover, seasonal trends in tissue-N content reflect both seasonal changes in seawater nitrogen availability and the ability of macroalgae to take up nitrogen in excess of growth demands and store it as cellular reserves (Mann, 1972; Hanisak, 1983). Second only perhaps to nitrogen bound in protein (Naldi and Wheeler, 1999), free (protein) amino acids (FAA) are considered to be an important storage pool that accumulates when excess nitrogen is supplied (McGlathery *et al.*, 1996; Naldi and Wheeler, 1999; Barr and Rees, 2003; Taylor *et al.*, 2006), and also one that is relatively quickly (over days) utilised when the external nitrogen supply is removed (McGlathery *et al.*, 1996; Liu and Dong, 2001).

Most of the research regarding the short-term changes in nitrogen storage pools in macroalgae is inferred from measurements of nitrogen uptake, typically that of surge (initial pool-filling) uptake (Rosenberg and Ramus, 1984; Harrison et al., 1986; Pedersen, 1994; Torres et al., 2004). However there are relatively few studies that have directly investigated changes in nitrogen storage pools (e.g. free amino acids, protein and total tissue nitrogen) in response to changes in external nitrogen supply

(Fujita *et al.*, 1988; McGlathery *et al.*, 1996; Naldi and Wheeler, 1999). Since rates of nitrogen uptake can often exceed the rate of growth in macroalgae (e.g. Hanisak, 1983) this has obvious implications for developing reliable indicators of nitrogen availability (in addition to nitrogen indices currently in use). In *Ulva intestinalis* it has been shown that the saturation of glutamine and glutamate coincides with the cessation of surge uptake (i.e. initial uptake that is thought to be due to filling of short-term storage pools) and the onset of internally controlled uptake (which is often used to infer the rate of ammonium assimilation) (Taylor *et al.*, 2006). In addition, there is considerable evidence that either the FAA pool, or at least some of its constituents, appear to function as short-term N-storage reservoirs in both microalgae (Dortch, 1982; Flynn and Al-Amoudi, 1988; Flynn *et al.*, 1994) and macroalgae (Pedersen, 1994; McGlathery *et al.*, 1996; Naldi and Wheeler, 1999; Barr and Rees, 2003) and may therefore represent an integration of recent N-supply. However, the time scale over which external nitrogen is integrated into the FAA pool is unknown. In addition, use of the term 'storage' with respect to the sum of free amino acids is complicated by the fact that its individual constituents are required for various metabolic and catabolic (e.g. photorespiration) processes, and under steady state growth, are continually incorporated into macromolecules and proteins in order to sustain growth.

Despite the fact that relatively little is known about accumulation of free amino acids in macroalgae in response to nitrogen concentration, this parameter has been successfully used to trace the influence of anthropogenic nitrogen in seawater (Jones *et al.*, 1996). It is known that the total FAA pool in macroalgae can make up a considerable percentage of the total tissue-N content, with values up to 47 % in red (Wilcox *et al.*, 2006), 55 % in green (McGlathery *et al.*, 1996) and 53 % in brown (Zimmermann and Kremer, 1986) algae. However, the range of the FAA pool within individual algal species can also vary with respect to its contribution to total tissue-N (TN). For example, the percentage contribution of FAA to TN can range from 8 – 45 % in *Chaetamorpha linum* (McGlathery *et al.*, 1996), 4 – 9 % in *Ulva fenestrata* (Naldi and Wheeler, 1999) and 7 – 70 % in *Gracilaria tikvahiae* (Bird *et al.*, 1982). While some of these changes may be due to the factors identified above (e.g. maintaining N-metabolism), there are no studies that have quantified the relationship

between amino acid content (including glutamine and glutamate) in macroalgae and sustained long-term nitrogen supply at ecologically relevant concentrations.

## 2 Marine pollution and eutrophication

### Background

The world's oceans, coasts and estuaries are the ultimate repository for a vast array of substances discharged either accidentally or deliberately via human activities (Kennish, 1997). While there is no precise definition of the term "pollution", one definition is: any stress on the natural environment caused by human activities resulting in an unfavourable change in an ecosystem (Kennish, 1997). According to Clark (1992), contamination in the marine environment occurs "when a man-made input increases the concentration of a substance in seawater, sediments, or organisms above the natural background level for that area and for the organisms". Marine contaminants are comprised of five main constituents (Sfriso *et al.*, 1991; Clark, 1992; Rainbow, 1995; Kennish, 1997; Kennish, 1998; Haritonidis and Malea, 1999; Wulff *et al.*, 2001):

- organic carbon enrichment related to elevated nutrient inputs, particularly nitrogen and phosphorus
- heavy metals associated with sewage effluents and sludges. These typically include cadmium, copper, lead, mercury and zinc.
- organochlorine compounds originating from the widespread use of domestic and agricultural herbicides, as well as various industrial wastes
- polycyclic aromatic hydrocarbons from industrial effluents, pyrolysis of organic matter, and other sources
- petroleum hydrocarbons from oil spills, sewage, and non-point source runoff

Contaminants may enter the sea via six primary pathways (Kennish, 1997):

- riverine
- non-point source runoff from land
- groundwater flow through the aquifer

- direct (reticulated) effluent discharges
- discharges and dumpings from ships
- atmospheric deposition

In recent years much attention has focused on the effects of pollution on marine mammals, fish and marine invertebrates, and the potential effects on humans as a consequence of the presence of contaminants in marine ecosystems. It is well established that persistent chemical compounds, often highly toxic, can affect whole ecosystems, particularly by accumulating in successively higher concentrations in higher trophic levels (a process commonly referred to as bioaccumulation). However, primary producers are also susceptible to the effects of persistent chemical compounds (often chlorinated) and heavy metals, which can be inhibitory factors affecting seaweed metabolism (Butterworth *et al.*, 1972; Knauer and Martin, 1972; Higgins and Mackey, 1987; Lobban and Harrison, 1997).

The effects of marine contaminants may be manifest in a numbers of ways, but generally take the form of a continuum ranging from chronic through to acute effects. The uptake of contaminants by marine animals occurs through the ingestion of food and detrital particles, water exchange at feeding and respiratory surfaces, and adsorption of chemicals onto the body surface (Kennish, 1997). Bioassay techniques have become an important part of marine pollution studies and are used to detect and assess changes in marine contaminants. Bioassays, using indigenous (if available) or "standardised" sensitive organisms, which are not usually indigenous to a study site, typically measure acute toxicity, sublethal effects, and genotoxicity in the laboratory. Standard bioassay organisms include mussels, oyster larvae, sea-urchin larvae and microalgae (Kennish, 1997).

One direct pathway for the entry of contaminants, and their subsequent accumulation in marine organisms is via primary producers – marine algae. The direct, short-term effects of environmental contaminants on marine (and freshwater) microalgae and macroalgae have been the subject of many laboratory studies (Fletcher, 1990). In more recent times there have been a growing number of studies that have examined accumulation of environmental toxins (e.g. heavy metals) in macroalgae (Fletcher, 1990; Sfriso *et al.*, 1991; Munda and Veber, 1996; Haritonidis and Malea, 1999;

Malea and Haritonidis, 2000). Much of this work has focused on the development of macroalgae as indicators of contaminant pollution, although some have suggested that interpretation of accumulated levels in macroalgae, such as *Ulva*, may be confounded by taxonomic differences (Rainbow, 1995). Irrespective of this, since they often form the base of many temperate coastal communities, there will be obvious potential for direct and indirect impacts on the communities that they support.

Sewage effluents and industrial wastewater discharges are obvious sources of a variety of pollutants including excessive concentrations of nutrients, particularly in the form of ammonium (Kennish, 1997; Valiela *et al.*, 1997). At high concentrations ammonium is toxic to marine invertebrates and fish, and can also inhibit seaweed growth at very high concentrations (> 30-50 µM) (Waite and Mitchell, 1972; Brockman *et al.*, 1988). The direct effects of pollution on macrophytes (e.g. inhibition of photosynthesis and growth (Moss and Woodhead, 1975; Hopkin and Kain, 1978)), and the cumulative effects of pollutants on macroalgae or seagrass-based ecosystems (Valiela *et al.*, 1997) are still relatively under-studied. It is known that increased nitrogen loading (mainly anthropogenic) is associated with both excessive growth of macroalgae in temperate estuaries (MacGregor *et al.*, 1995; Valiela *et al.*, 1997) and tropical coral reefs (McClanahan *et al.*, 2003) alike, and also with the demise of seagrass beds (Valiela *et al.*, 1997) or in some cases increases in the extent of seagrass beds (Udy *et al.*, 1999).

In addition to nutrient inputs from point sources there is also concern about the effects of fertiliser nutrients on aquatic ecosystems (Galloway *et al.*, 2003). Although priority lists of problem pollutants tend to focus on persistent chemical compounds (e.g. polychlorinated biphenyls, polycyclic aromatic hydrocarbons, chlorinated pesticides (including DDT) and various heavy metals) (Kennish, 1998), nutrient pollution has also been considered to be of major concern (McIntyre, 1992). Moreover, in more recent reviews eutrophication, which is often linked to nutrient enrichment (Wulff *et al.*, 1990), is now considered by some to be the second highest threat to estuarine ecosystems below that of habitat loss (Kennish, 2003).

**Nutrient enrichment and eutrophication in the marine environment**

The eutrophication process characterises one of the central themes in freshwater and marine ecology: the production of organic matter that forms the basis of aquatic food webs (Livingston, 2001). However, the concept of marine eutrophication was unheard of until relatively recently (Wulff *et al.*, 1990), but it is now recognised as one of the main threats to coastal ecosystems (Wulff *et al.*, 1990; Cloern, 2001). Moreover, it is now known that nutrient enrichment is one of the main contributing factors to eutrophication (Brockman *et al.*, 1988; Wulff *et al.*, 1990; Nixon, 1995; Nixon, 1998; Cloern, 2001; Turner and Rabalais, 2003).

Eutrophication is broadly defined as an increase in the amount of energy, production or organic matter to receiving waters. For example, a trophic state definition is based on the amount of organic carbon supply delivered to the system (Nixon, 1995):

| | ($g\ C \cdot m^{-2} \cdot y^{-1}$) |
|---|---|
| Oligotrophic | < 100 |
| Mesotrophic | 100 - 300 |
| Eutrophic | 301 - 500 |
| Hypertrophic | > 500 |

It was only about four decades ago that explanations for eutrophication processes in marine systems were broadly based on our understanding of nutrient responses in freshwater systems, which relied on the empirical relationship between an increase in the supply of nutrients and a predictable response by the system (e.g., changes in primary production, chlorophyll and dissolved oxygen) (Cloern, 2001). However, since then there has been an increasing awareness that eutrophication in marine systems is a complex process in which inputs are not necessarily good predictors of system responses (Nixon, 1995; Cloern, 2001). A combination of biological, chemical and physical factors (particularly tidal flushing) are now known to play important roles in determining the responses of marine systems to nutrient loading, particularly in more sheltered, shallow environments (Cloern, 2001).

Phosphorus and nitrogen are critical macronutrients for micro- and macroalgae, but nitrogen is generally thought to be the major limiting nutrient during peak seasonal growth of macroalgae in temperate regions of the world (Hanisak, 1983). However, elevated nitrogen (and phosphorus e.g., Carpenter *et al* (1998) and Nixon (1998)) concentrations in seawater can also adversely affect aquatic (both freshwater and marine) environments, and are often derived from sewage (including seepage from septic tanks), industrial effluents, or agricultural fertiliser runoff. Sewage effluents are often associated with high concentrations of dissolved inorganic nitrogen, particularly ammonium (Kennish, 1997), although elevated levels of nitrate in groundwater are often associated with wastewater and septic tank discharges (Valiela *et al.*, 1997).

Excessive growth or changes in growth patterns of marine primary producers can often reflect changes in the coastal environment (Brockman *et al.*, 1988; Nixon, 1995; Valiela *et al.*, 1997; Nixon, 1998; Schramm, 1999; Howarth *et al.*, 2000; Cloern, 2001). These changes are often linked to increasing urbanisation or changes in land-use practises (Valiela *et al.*, 1997; Schramm, 1999). The term "algal bloom" tends to be synonymous with microalgae that can occur in such high densities that they are recognised by a change in the colour of the water (e.g. red tides). However, extensive, and sometimes monospecific, blooms of macroalgae can indicate eutrophication, often due to anthropogenic nutrients (Sfriso *et al.*, 1991; Nixon, 1995; Morand and Briand, 1996; Valiela *et al.*, 1997; Schramm, 1999). In severe cases it may lead to hypertrophication (Morand and Briand, 1996; Schramm, 1999). The main macroalgae associated with these proliferations are members of the opportunistic genera, *Chaetomorpha*, *Cladophora*, *Gracilaria* and *Ulva* (Lavery and McComb, 1991; Morand and Briand, 1996; Valiela *et al.*, 1997; Schramm, 1999). Such proliferations are typically associated with polluted estuaries or semi-enclosed waters, but have been recorded in several large harbours around the world (Lavery and McComb, 1991; Sfriso *et al.*, 1991; Sfriso and Pavoni, 1994; Morand and Briand, 1996; Valiela *et al.*, 1997; Schramm, 1999). New Zealand examples include nuisance blooms of *Ulva* in both Tauranga Harbour (Hawes *et al.*, 1992) and the Avon Heathcote Estuary, Christchurch (Steffensen, 1976).

Coastal environments can often be affected by anthropogenic sources of nutrients such as those associated with municipal sewage/wastewater discharges, particularly

those located in more sheltered situations (Rosenberg, 1985; Nixon, 1995; Nixon, 1998; Cloern, 2001; Smith *et al.*, 2003). However, there is also evidence of eutrophication processes operating at larger scales than previously thought likely or even possible (Nixon, 1998; Turner and Rabalais, 2003; Dybas, 2005). Until about four decades ago the oceans, partly because of their sheer size, were thought to be immune from the effects of nutrient enrichment and oxygen depletion (Nixon, 1998). It is clear that this assumption was incorrect and it is now known that nutrient loading to the world's oceans has increased significantly over the last three decades (Smith *et al.*, 2003). Examples of large water bodies which have tended towards eutrophication as a result of human activities, are the Baltic and Black Seas and the northern Gulf of Mexico (Wulff *et al.*, 1990; Nixon, 1998; Wulff *et al.*, 2001; Smith *et al.*, 2003). The latter example has been well-studied and the large-scale effect of anthropogenic nutrients has been implicated (Howarth *et al.*, 2000; Turner and Rabalais, 2003; Dybas, 2005). Each summer the bottom water layer becomes anoxic to a point where it cannot support most marine life. Partly dependent on climatic conditions (particularly wind and rain), in an area centred on the Mississippi delta, this anoxic layer regularly increases in size to over 15,000 $km^2$ (Dybas, 2005). Although it is known that these events have occurred at least since the 1800's, there is now evidence that they have become more extreme since the 1950's when farmers began using fertilisers more intensively (Dybas, 2005).

Anoxia occurs as a result of excessive primary (phytoplankton) productivity leading to increased rates of decaying matter reaching the bottom layer which in turn results in increasing rates of oxygen demand by decomposers. In the summer the problem is exacerbated by thermocline formation, which isolates bottom waters from surface oxygen re-supply (Turner and Rabalais, 2003; Dybas, 2005). One of the main factors responsible for the excessive primary production in the Gulf of Mexico is the input of nutrients into the Mississippi river from its various watersheds. It is now thought that fertiliser application in intensive agricultural areas and associated soil disturbance and loss ultimately contribute significant amounts of nutrients (1.6 million tons of nitrogen and 100,000 tons of phosphorus since 1980) into the northern Gulf of Mexico (Dybas, 2005). It has been suggested that there are now 146 such areas around the world (43 in the USA) that are now often referred to as marine dead zones

(Dybas, 2005), although it should also be noted that scientific corroboration of this number is sparse.

In the 1990's more synthetic fertiliser was used globally than had been used in the entire previous history of agriculture (Nixon, 1998). In New Zealand, total fertiliser consumption increased by around 170 percent in the period 1990 to 1999 compared with an increase of seven percent for the OECD countries. However, the use of nitrogen in fertiliser has grown by over 500 percent over a similar period (Saunders and Ross, 2004). Although large scale impacts to our coastal environments from excessive nutrient inputs may seem improbable (just as perhaps they did to those considering the potential for eutrophication in the oceans only a few years ago) the potential threat to New Zealand's marine ecosystems posed by increases in nutrient levels deserves serious consideration. As Nixon (1998) states, "with large stretches of coastline exposed to unprecedented levels of nitrogen, it seems inevitable that ocean waters around the world will become greener, browner and redder and that there will be more frequent periods when the bottom of the sea in vulnerable locations becomes lifeless".

**Monitoring changes in nutrients in the marine environment**

Monitoring changes in the concentration of nutrients in coastal environments has historically been done by direct measurement in seawater column samples. However, there may be little correlation between water column nitrogen (or phosphorus) concentrations and either productivity or growth of primary producers (Fong and Zedler, 1993; Fong *et al.*, 1998). This may be due to analytical measurements not reflecting the bioavailable concentrations of nutrients (Lyngby, 1990). Further, because of the abilities of micro- and some macroalgae to take up nutrients rapidly, the ability to make predictions based on water column nutrient measurements may be limited, particularly when systems are subject to transient influxes of nutrients (Björnsäter and Wheeler, 1990; Jones *et al.*, 1996; Valiela *et al.*, 1997; Fong *et al.*, 1998). Moreover, changes in nutrient concentrations may also be attributed to the uptake abilities of the primary producers themselves (Flindt *et al.*, 1999), particularly when they are present in high biomass (Thybo-Christesen *et al.*, 1993).

Physical and chemical processes (and ecology) of shallow estuarine systems are typically and often strongly influenced by freshwater runoff from the land, and tidal exchange of water with the adjacent open sea (Flindt *et al.*, 1999; Pereira *et al.*, 2001). Physical forcing by tides and winds in shallow estuaries can result in the rapid replacement of more saline, nutrient-poor marine water with less saline, nutrient-rich estuarine water (Stumpf *et al.*, 1993). Tidal exchange is probably the more dominant influence on nutrient concentrations in harbour systems that don't have large freshwater inflows (Williams and Rutherford, 1983, Auckland Regional Authority, 1983 #1043). In addition, nutrient concentrations can vary in space and time as a result of other physical, chemical and biological processes. For example it has been shown that ammonium efflux from sediments can be considerable at low tide after they have been exposed to the air (Hou *et al.*, 2005) while differences in nutrients can also be associated with diel period and depth (Sakamaki *et al.*, 2006). Given that nutrients can vary as a result of a wide range of factors it might be concluded that the instantaneous measurements of seawater nutrients alone may yield little information on what is actually available to photoautotrophs, especially on a time average basis. Since the temporal and spatial resolution of nutrient measurements is limited by traditional water sampling, *in situ* nutrient autoanalyser technologies have been developed for use in coastal monitoring (Hanson and Moore, 2001). However, while these technologies undoubtedly provide valuable information they may often be too costly for wide-scale use.

## Macroalgae as indicators of nutrient enrichment and marine pollution

### *Biochemical aspects*

Partly as a result of the limitations and possibly the cost of conventional water monitoring, increasing attention has been given to macroalgae as bioindicators of changes in water column nutrient levels (Atkinson and Smith, 1983; Björnsäter and Wheeler, 1990; Al-Amoudi, 1994; Wilkinson *et al.*, 1994; Jones *et al.*, 1996; Fong *et al.*, 1998; Barr and Rees, 2003). While measurement of seawater chlorophyll content is a standard practise for monitoring changes in phytoplankton productivity (Strickland and Parsons, 1972; Thomas *et al.*, 1974) and, by inference, potential changes in nitrogen (nutrient) availability, there are relatively few studies on macroalgae that link levels of chlorophyll and accessory pigments to levels of N-loading (Lapointe and Tenore, 1981; Bird *et al.*, 1982; Jones *et al.*, 1996; Barr and

Rees, 2003). To date most attention has been given to N-status in macroalgae, since nitrogen is generally considered to be the primary limiting nutrient (Hanisak, 1983). This has often been inferred from rates of nitrate and ammonium uptake in relation to seawater N-loading (Peckol and Rivers, 1995; McGlathery *et al.*, 1996; Campbell, 1999). However, many have also advised caution when interpreting the ecological and physiological significance of uptake data (Hanisak and Samuel, 1983; Harrison *et al.*, 1989; Pedersen, 1994; Rees *et al.*, 1998).

Until fairly recently total N-content (or C : N) was thought to be the only reliable indicator of N-status in common use in studies of microalgae (Flynn *et al.*, 1989) and macroalgae (Atkinson and Smith, 1983; Björnsäter and Wheeler, 1990; Al-Amoudi, 1994; Peckol *et al.*, 1994; Jones *et al.*, 1996; Fong *et al.*, 1998). However, it is generally thought that N-content may be more indicative of the plant's recent nutritional history and, as such, is less likely to reflect changes in N-availability occurring over short (hours) time scales (Flynn and Fielder, 1989). Moreover, seasonal fluctuations in N-content in natural macroalgal populations have been demonstrated (Chapman and Craigie, 1977; Rosenberg and Ramus, 1982; Wheeler and Weidner, 1983; Thomas and Harrison, 1985) which would tend to confound interpretation of tissue-N in relation to N-loading. Similarly, small-scale spatial distribution patterns of seaweeds have been attributed to differential nitrogen acquisition resulting in differences in tissue-N content (Thomas and Harrison, 1987). Irrespective of these patterns, the use of tissue-N content in macroalgae has been successfully applied as a bioindicator of N-loading (Peckol *et al.*, 1994; Jones *et al.*, 1996; Fong *et al.*, 1998; Barr and Rees, 2003). However, there have been few studies investigating the potential role of amino acid levels as indicators of nitrogen availability in macroalgae (Jones *et al.*, 1996; Barr, 2000; Jones *et al.*, 2001; Barr *et al.*, In preparation).

***Eco-physiological aspects***

While nitrogen indices in natural populations of macroalgae can clearly indicate differences between contrasting environments (Fong *et al.*, 1998; Barr and Rees, 2003) or within environmental gradients (Jones *et al.*, 1996; McClelland *et al.*, 1997; Rogers, 1999) it is likely that biochemical changes in algal indices will be confounded by a variety of other factors in the environment. In addition to seasonal effects, it

might also be difficult to attribute biochemical responses in macroalgae to specific causal factors (e.g. nitrogen concentration) because of differences in confounding variables between environments or because of uncertainty of comparisons between different taxonomic groups (e.g. *Ulva* (Rainbow, 1995)). As a result it might be difficult to link contrasting environmental effects to responses in different populations of macroalgae covering a large environmental or geographic range.

One way some of these issues could be addressed is to use standardised test-algae which can be deployed into different environments under examination. The utility of macroalgae in field transplantation experiments has been demonstrated in many recent studies of macroalgae (Horrocks *et al.*, 1995; Jones *et al.*, 1996; Fong *et al.*, 1998; Barr, 2000; Costanzo *et al.*, 2000; Costanzo *et al.*, 2001; Jones *et al.*, 2001; Aguiar *et al.*, 2003). In most cases these studies have tended to focus on nitrogen loading (and source, in the case of stable nitrogen isotopes (see below)) generally in experiments using environmental gradients. However, one of the underlying assumptions in this approach is that responses of indicator species are due to changes in the environmental gradient under investigation and not to changes in the physiological responses to other factors (e.g. light, temperature, water motion, taxonomic differences).

Historically phytoplankton, together with various marine macrofauna (particularly benthic invertebrates), have received most attention as test-organisms and indicators of coastal water quality (Levine, 1984). However, while there have been misgivings about the insensitivity of macroalgae to some chemical pollutants (Rainbow, 1995), there is also the view that they have distinct attributes that make them ideal indicator and/or test organisms (Fletcher, 1990). One advantage is that seaweeds are mostly conspicuous and samples are easily collected. In contrast to phytoplankton populations that will consist of many species, which are either difficult or impossible to identify, macroalgae are generally much easier to identify to species level and it is possible to sample individuals in a given population. Coupled with this, seaweeds are sessile and often perennial (Lobban and Harrison, 1997), which means they have the ability to accumulate contaminants, such as heavy metals, over long periods of time at one site (Fletcher, 1990).

In addition to their ability to reflect nitrogen availability, macroalgae also reflect levels of heavy metals in the environment (Luoma *et al.*, 1982; Munda and Veber, 1996; Haritonidis and Malea, 1999; Wang and Dei 1999; Malea and Haritonidis, 2000; Villares *et al.*, 2001; Gosavi *et al.*, 2004; Strezov and Nonova, 2005). Moreover, some opportunistic genera, such as *Ulva*, as well as being hardy are also cosmopolitan allowing results from different regions to be compared (Fletcher, 1990). These qualities, as Levine (1984) points out, are paramount criteria for successful monitoring and make some seaweeds ideally suited as test-organisms. Moreover, although significant changes in structure of phytoplankton populations may be the end-product of increasing nitrogen levels, macroalgae are generally the first group of organisms to respond (Valiela *et al.*, 1997). Consequently, it is suggested that macroalgae may be better indicators of the early stages of N-enrichment.

**Natural abundance stable nitrogen isotopes in macroalgae**

Nitrogen exists as two naturally occurring stable isotopes, $^{14}N$ and $^{15}N$. All N-containing compounds on earth show a $^{14}N$ / $^{15}N$ ratio of close to 273 (Heaton, 1986). Since the abundance of $^{15}N$ is small compared to $^{14}N$, the relative amount of $^{15}N$ is expressed (with reference to an atmospheric air standard) in parts per thousand (‰) using δ notation as follows:

$$\delta^{15}\mathrm{N}\,(‰) = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1000$$

, where $R_{\text{sample}}$ and $R_{\text{standard}}$ are molar ratios of $^{15}N$ / $^{14}N$.

The $\delta^{15}N$ values of a product and its substrate in a biological or chemico-physical process will be different depending on the relative reaction rates of $^{14}N$ and $^{15}N$. This is called the isotopic effect in which the rate constant (a measure of the reaction rate) for $^{14}N$ is typically higher than $^{15}N$. It is expressed as the ratio of the rate constants ($k_{14}$ / $k_{15}$) and is typically > 1 (Fry, 1970). In biological systems each step in the sequence of processes will have its own kinetic isotopic effect (Shearer and Kohl, 1986). However, in many cases it is more practical to consider the sum of all of the steps; the observed overall fractionation of an organism (Bedard-Haughn *et al.*, 2003).

The use of natural abundance stable nitrogen isotopes has become increasingly common in recent years for tracing the source, flow and fate of nitrogen (particularly of anthropogenic origin, e.g., sewage or agricultural fertiliser) in aquatic environments (Heaton, 1986, Bedard-Haughn, 2003 #997). There are now a number of studies that have used nitrogen isotopes to investigate linkages between anthropogenic sources of nitrogen in the marine environment and its influence on primary producers (typically macroalgae and seagrasses) (McClelland *et al.*, 1997; Rogers, 1999; Jones *et al.*, 2001; Umezawa *et al.*, 2002; Rogers, 2003, Savage, 2004 #976; Cohen and Fong, 2005). In addition, nitrogen isotopes (often in combination with carbon ($\delta^{13}C$) and even sulphur ($\delta^{34}S$) isotopes) have been used to trace the flow of organic matter through trophic levels (Peterson *et al.*, 1985; McClelland *et al.*, 1997; Rogers, 1999; Davenport and Bax, 2002; Schmidt *et al.*, 2004; Vizzini and Mazzola, 2004; Pruell *et al.*, 2006; Vizzini and Mazzola, 2006) in marine ecosystems. It is generally thought that fractionation occurs between trophic levels, which increases $\delta^{15}N$ by about 3 ‰ between each succeeding trophic step (i.e. tissue nitrogen is enriched in $^{15}N$ in each succeeding trophic step) (Minagawa and Wada, 1984; Keough *et al.*, 1996).

One of the requirements for the natural abundance $\delta^{15}N$ method in tracing anthropogenic nitrogen in marine mixing zones is that the signature of the suspect source must have a $\delta^{15}N$ sufficiently distinct from the background values (i.e. that deemed to be characteristic of natural origin) to allow resolution. For example, studies in some coastal environments have shown that $\delta^{15}N$ values in seawater DIN and *Ulva* tissues greater than about 9 ‰ (Jones *et al.*, 2001; Gartner *et al.*, 2002; Dudley, 2005) may indicate the influence of secondary or tertiary treated sewage nitrogen while other studies have shown that untreated wastewater sources can result in ratios approaching 2 ‰ (Rogers, 1999; Dudley, 2005). However, background $\delta^{15}N$ will also vary depending on the different chemical, physical and biological interactions in a given environment. In the case of the studies above, the background (natural) $\delta^{15}N$ values lay between 2 and 9 ‰. However, in other studies such as those in harbours with large watersheds, background $\delta^{15}N$ values may be largely controlled by ground water nitrate with $\delta^{15}N$ values approaching 0 ‰ (McClelland and Valiela, 1998; Bedard-Haughn *et al.*, 2003).

An additional constraint of the natural abundance method is that $\delta^{15}N$ of source values are typically required for the relative quantification of nitrogen contributions, in turn inferred from mixing models such as proposed by Spies *et al* (1989). The natural $\delta^{15}N$ abundance method is therefore best considered as a semi-quantitative parameter (Bedard-Haughn *et al.*, 2003). In addition, many advise caution when interpreting isotope patterns since many factors can affect the relative amounts of $^{14}N$ and $^{15}N$ in both organisms and the environment as a whole (Heaton, 1986; McClelland *et al.*, 1997; Bedard-Haughn *et al.*, 2003; Schmidt *et al.*, 2004; Cohen and Fong, 2005; Vizzini and Mazzola, 2006). For example, there is evidence that suggests $\delta^{15}N$ patterns in different primary producer and consumer groups can show considerable spatial variation (Vizzini and Mazzola, 2006) as well as variation within trophic (e.g. primary or secondary consumers) and taxonomic groups (Vanderklift and Ponsard, 2003).

It is generally thought that $\delta^{15}N$ signatures in primary producers closely reflect the $\delta^{15}N$ of DIN. This may be especially true for algae since nitrogen is taken up (along with other nutrients) directly from the water column. Many studies that have focussed on macroalgae have demonstrated strong links between isotopic composition of source nitrogen and that of tissue (McClelland and Valiela, 1998; Rogers, 1999; Savage and Elmgren, 2004). Therefore it is not surprising that there is an implicit assumption that macroalgae assimilate $^{14}N$ and $^{15}N$ in roughly the same proportions as those of the source pool (i.e. there is little or no fractionation). However, studies on microalgae have shown that $^{15}N$ fractionation in diatoms may be considerable with values as high as 20 ‰ for ammonium enrichment and 5 ‰ for nitrate enrichment (Waser *et al.*, 1998). In contrast it has also been shown that $^{15}N$ fractionation may be less in dinoflagellates with values in the order of 1-3 ‰ fractionation (Montoya and McCarthy, 1995) although others have shown that there is no clear pattern in fractionation between different microalgae groups (Needoba *et al.*, 2003). In addition, fractionation in microalgae can also be affected by factors such as light (Needoba and Harrison, 2004).

In contrast to studies involving microalgae, there are virtually none examining fractionation processes in macroalgae (Cohen and Fong, 2005), despite the large number of studies that utilise $\delta^{15}N$ signatures in macroalgae. In the case of at least

*Ulva* (sym. *Enteromorpha*) *intestinalis* one study has reported that there may be little or no fractionation under ammonium or nitrate enrichment, or as result of concentration differences (Cohen and Fong, 2005).

## 3 Biology of *Ulva* (Ulvaceae, Ulotrichales, Chlorophyta)

### *Ulva* taxonomy

*Ulva* was one of the first Linnaean genera (Hayden *et al.*, 2003). It is traditionally described as a green alga with flattened frondose thalli divided into elongated segments, sometimes spirally twisted with crisp margins (Adams, 1994) (Plate 1.1). *Ulva* and *Enteromorpha* are morphologically distinct and until recently were classified as two separate genera. Frondose *Ulva* have thalli consisting of two adhered cell (distromatic) layers while *Enteromorpha* have tubular thalli consisting of one cell (monostromatic) layer. Based on recent DNA analysis *Enteromorpha* has now been reduced to synonymy with *Ulva* (Hayden *et al.*, 2003).

**Plate 1.1**. *Ulva fasciata* showing spirally twisted thalli

It is a cosmopolitan genus with 547 species names in the AlgaeBase species database at present, of which 100 species (including at least 35 *Enteromorpha* species) are currently recognised world-wide (Tanner and Wilkes, 2005). In New Zealand, many more forms and varieties of *Ulva* have been recorded (Adams, 1994; Heesch, 2006). However, more recent investigations into New Zealand's *Ulva* diversity suggests that there are at least 25 species although many of these have not yet been formally described (Heesch, 2006). It is also probable that some *Ulva* species have been introduced into New Zealand waters in recent times (Adams, 1994; Heesch, 2006). By traditional classification some common members of Chlorophyta in New Zealand include: *Enteromorpha intestinalis, E. compressa, Ulva fasciata, U. lactuca, U. rigida, U. scandinavica* and *U. spathulata* (Adams, 1994). However, given the recent research into New Zealand's *Ulva* diversity, it is very likely that this picture will change significantly in the near future.

**Life history and ecology of *Ulva***

In *Ulva,* when haploid *and* diploid phases occur they result in morphologically similar (isomorphic) gametophyte and sporophyte stages, respectively (Raven *et al.*, 1992; Lobban and Harrison, 1997). Sexual reproduction in *Ulva* generally occurs with fertilisation of free biflagellate anisogamates released from the surface of mature distal thalli. Similarly, asexual propagation occurs as a discharge of biflagellate or quadriflagellate zoospores from the thallus surface leaving conspicuous non-pigmented patches (Adams, 1994). Reproduction may also occur solely by parthenogenic gametes or zoospores (Poole and Raven, 1997).

*Ulva* is an opportunistic genus that is typically found in intertidal and estuarine habitats (Adams, 1994; Blomster *et al.*, 1998); however it has a wide variety of growth strategies and, as some suggest, is a successful "Jack-of-all-trades in the seaweed world" (Vermaat and Sand-Jensen, 1987). Several characteristics enable *Ulva* (and *Enteromorpha*) to dominate a variable environment: it is both euryhaline and eurythermal, desiccation and temperature-tolerant, and has a relatively low light saturation of photosynthesis (Vermaat and Sand-Jensen, 1987; Fong and Zedler, 1993; Poole and Raven, 1997; Fong *et al.*, 1998). However, as well as being common in sheltered environments *Ulva* can also occur on exposed coastlines. Its adaptability is demonstrated by recent "invasion" of freshwater habitats by some species of *Ulva*

(Poole and Raven, 1997). Interestingly however, earlier work by Vermaat and Sand-Jensen (1987) demonstrated that *Ulva lactuca* was able to persist under ice for extended periods during the Danish winter in Roskilde Fjord.

In comparison with most other macroalgae, *Ulva* is fast growing (Björnsäter and Wheeler, 1990), has a high SA:V ratio (Rosenberg and Ramus, 1984; Taylor *et al.*, 1998; Taylor *et al.*, 1999), has high rates of ammonium uptake and a large storage capacity for ammonium / nitrogen (Thomas and Harrison, 1987; Taylor *et al.*, 1998; Taylor *et al.*, 1999). *Ulva* has limited storage capacity for nitrogen (Fujita, 1985) and little defence against herbivores (Lobban and Harrison, 1997), other than perhaps its high growth rate. It displays a high degree of physiological and morphological plasticity in a wide variety of environments. Moreover, *Ulva* is a cosmopolitan genus, which is ubiquitous and capable of forming nuisance blooms. The linkage between the *Ulva* proliferation adjacent to urban environments and sewage pollution can be traced back as far as the 1900's (Steffensen, 1976; Blomster *et al.*, 1998). These characteristics therefore make *Ulva* a logical candidate as a biological indicator not only in an ecological sense, but also potentially in terms of its biochemical response to changes in the environment.

## 4 Aims of thesis

The aims of this thesis are to investigate the potential of *Ulva* as an indicator of N-loading. This was investigated using three separate approaches as follows :

**Chapter Two** Geographic survey of nitrogen status in natural *Ulva* populations from a wide range of environments around New Zealand, in summer and winter.

**Chapter Three** Experimental assessment of responses of nitrogen indices in *Ulva* to nitrogen concentration, nitrogen source, light and seawater motion.

**Chapter Four** Development and evaluation of test-*Ulva* (i.e., that of a standardised nutrient status), which was deployed into a range of marine environments around Auckland. With monthly sampling and replacement, the nitrogen status in test-*Ulva* was followed for one year.

# Chapter Two

# Geographical variation in nitrogen status of New Zealand *Ulva*

## 2.1 Abstract

To investigate nitrogen relationships in natural *Ulva* populations, 32 sites situated in a range of environments around New Zealand were haphazardly sampled during the summer and winter of 2002. *Ulva* samples were collected at low tide from four main environmental categories: sheltered rural, exposed rural, sheltered urban and exposed urban sites. In addition, there were two other categories: nitrogen-enriched urban sites and rural rock pools. Collections focused mainly on frondose *Ulva* species including *U. pertusa*, *U. fasciata*, *U. scandinavica* and *U. spathulata*, although *U. intestinalis* was also collected. Nitrogen-indices (including levels of free amino acids, chlorophyll *a*, chlorophyll *b*, tissue nitrogen and $\delta^{15}N$) from all *Ulva* samples collected were measured and compared with seawater nutrient concentrations.

Seawater nutrient concentrations were highly variable between all sites in both summer and winter. However, in the summer enriched urban sites had the highest mean total inorganic nitrogen (TIN) concentrations and *Ulva* with the highest mean levels of all N-indices compared with any other environmental category. Sheltered rural sites (those with no supposed strong influence of anthropogenic nitrogen) had the lowest levels of most N-indices measured in *Ulva*. In the winter, *Ulva* contained more nitrogen (reflected in all N-indices) compared with *Ulva* in the summer, particularly when growing in colder southern seawater on more exposed coasts. From combined summer and winter data, *Ulva* growing on exposed coasts showed an average increase in tissue nitrogen (TN) of 1356 μmol · g $DW^{-1}$ with a 10 ˚C decrease in seawater temperature. From statistical ordination, three groupings of *Ulva* were identified in both summer and winter; those of low, medium and high nitrogen-status. Between summer and winter surveys, *Ulva* growing in sheltered sites within the three ordination groups

showed similar correlative relationships between TN content and seawater temperature. For *Ulva* growing in sheltered environments, there was average increase of 585 μmol · g $DW^{-1}$ TN content with a 10 ˚C decrease in seawater temperature. It was concluded that TN content in *Ulva* was strongly influenced by interactive effects of external (seawater) nitrogen concentration, temperature (and therefore probably light) and relative water motion.

Stable nitrogen isotopes ($\delta^{15}N$) in *Ulva* from the enriched urban category had the widest range (4.77 ± 0.04 ‰ to 15.16 ± 0.03 ‰) of values compared with all other categories in both summer and winter. Conversely, *Ulva* from exposed rural sites had the lowest range of $\delta^{15}N$ values compared with any other category (6.7 ± 0.1 to 8.8 ± 0.1 ‰) with means values of 7.8 ‰ (± 0.6 s.d.) and 7.6 ‰ (± 0.6 s.d.) for summer and winter, respectively. There was no statistically significant seasonal difference in $\delta^{15}N$ values from either rural or urban sites. The only statistically significant difference in any *Ulva* N-indices between rural and urban categories (excluding *Ulva* from the enriched urban sites) was in tissue $\delta^{15}N$ values. It was therefore concluded that the combination of both $\delta^{15}N$ values (as qualitative measures in the context of this survey) and the quantitative N-indices in *Ulva* may be useful in investigations of both nitrogen source and nitrogen loading.

## 2.2 Introduction

Some of the elements required to maintain the main metabolic processes in macroalage can be found in seawater in relative abundance, while others, such as phosphorus and nitrogen, may often be found in low concentrations. Of all the limiting nutrients, nitrogen (N) has probably received the most attention and in temperate coastal environments nitrogen availability may frequently limit the rate of growth and / or yield of seaweeds (Mann, 1972; Hanisak, 1983; Smith, 1984). Sources of inorganic nitrogen in seawater are often present in low concentrations (Sharp, 1983). The occurrence of upwelling in coastal areas can dramatically increase inorganic nitrogen levels, with subsurface nitrate levels approaching 30 μM (Hanisak, 1983). Nitrate influxes to estuaries and nearshore coastal environments are also derived from rivers

(Lavery and McComb, 1991). Moreover, the process of catchment urbanisation can lead to increases in nitrogen loading to riverine systems (Valiela *et al.*, 1992; McClelland *et al.*, 1997; Schramm, 1999) and can often be linked to other human activities (e.g., agricultural runoff) (Dybas, 2005). Ammonium is another important source of nitrogen for macroalgae although it is typically found in low concentrations in natural seawater. However, high concentrations in seawater can result from direct discharge of sewage and industrial effluents to receiving marine environments.

It is well known that macroalgae can and do derive nitrogen from a variety of sources (both natural and anthropogenic in origin such as those listed above), and it is generally thought that levels of tissue nitrogen reflect environmental concentrations in seawater. Until recently, total tissue-N (TN) content or the ratio of nitrogen : carbon (N : C) was the only reliable indicator of N-content in microalgae (Flynn *et al.*, 1989) and macroalgae (Atkinson and Smith, 1983; Björnsäter and Wheeler, 1990; Al-Amoudi, 1994; Peckol *et al.*, 1994; Jones *et al.*, 1996; Fong *et al.*, 1998). However, seasonal fluctuations in the N content of seaweeds have been demonstrated for some natural populations (Chapman and Craigie, 1977; Rosenberg and Ramus, 1982; Wheeler and Weidner, 1983; Thomas and Harrison, 1985), and it is now known that, in addition to the effects of seawater nitrogen loading (Fong *et al.*, 1998), nitrogen content is also influenced by temperature (Rosenberg and Ramus, 1982; Duke *et al.*, 1987; Duke *et al.*, 1989a; Altamirano *et al.*, 2000) and light (Duke *et al.*, 1987; Duke *et al.*, 1989a; Altamirano *et al.*, 2000). Other recent studies have also suggested that the pool of free amino acids (and its individual constituents, such as glutamine or asparagine) may also be a useful measure of nitrogen availability (Jones *et al.*, 1996; Barr and Rees, 2003; Taylor *et al.*, 2006).

The opportunistic green alga *Ulva* displays a high degree of physiological and morphological plasticity in a wide variety of environments. Moreover, *Ulva* is a cosmopolitan genus and is common around New Zealand in a variety of environments ranging from rock pools, estuaries and harbours, to exposed coasts and offshore islands. The environments in which *Ulva* grows are often characterised by substantial fluctuations in abiotic variables (including nutrient concentrations, light and temperature), which will change over different time scales. However, little is known about large scale geographic patterns in nitrogen content in *Ulva*. It might be expected

that environments that contrast in levels of seawater nitrogen loading might also produce *Ulva* that also contrast in levels of tissue-N. In addition, given that nitrogen metabolism is fundamental to algae it might be expected that at least some biochemical N-indices of nitrogen availability in *Ulva* should over-ride the effect of genetic (species) differences. In order to examine these suggestions a survey of both N-indices in *Ulva* and seawater nitrogen concentrations from environments that were likely to contrast in levels of nitrogen availability was conducted in the summer and winter of 2002.

The main aims of this survey were two-fold:

1) To examine levels of free amino acids, chlorophyll, and total tissue nitrogen (and $\delta^{15}N$) content in *Ulva* in relation to contrasting concentrations of seawater nitrogen, and to contrasting physical environments in both urban and rural settings.
2) To examine contrasting seasonal differences in the nitrogen status in *Ulva* by comparing the same populations (as far as possible) in summer and winter. The surveys were conducted in February – March to coincide with seasonal seawater temperature maxima, and August – September to coincide with seasonal seawater temperature minima (Evans, unpublished data).

## 2.3 Methods

### 2.3.1 Survey sites and environmental categories

Two surveys of *Ulva* populations in New Zealand were conducted in summer and winter of 2002. The first survey was carried out in mid to late summer (January / February) 2002, and the second in mid to late winter (August / September) 2002. Frondose *Ulva* species were collected from 27 haphazardly selected sites in the summer and 29 sites in the winter (Figure 2.1 and Table 2.1. Also see Appendix 2.1 for collection dates and site grid map co-ordinates). In the winter survey there were three sites where *Ulva* was absent where it had been present in the summer. These were Mount Maunganui (MM), Humber Road (HU) and South Spit Road (SS). In addition, five sites were added to the winter survey (Table 2.1).

The sites surveyed fell into 6 broad subjective categories:

1) Sheltered rural sites. These were embayments or estuaries and were largely uninhabited. Theoretically these sites were not likely to have been affected by large inputs of sewage or industrial effluents, but may have been subject to either agricultural (e.g. fertilisers) or farm septic-tank runoff. There were two sites in this category in the summer, Cable Bay (CA) near Nelson and Inner Beach (IN) at the eastern base of Farewell Spit. Hooper's Inlet (HO) on the Otago Peninsula was added to these two in the winter survey (Figure 2.1 and Table 2.1).

2) Exposed or semi-exposed rural sites. These were typically in more open coastal areas which were unlikely to have been influenced by significant urban nutrient inputs. There were eight sites with the most northern at the Mokohinau Islands (MK) and most southern at Taieri (TI) adjacent to an estuary mouth.

3) Rock-pool populations in rural, open coastal sites. These were high intertidal rock pool *Ulva* populations and were represented by a northern site on the east coast of the North Island (Mermaid Pools (MP)), and a southern site on the west coast of the South Island (Charlston (CH)). At both these sites the rock pools were also well populated by mussels (*Xenostrobus pulex*), which would excrete ammonium that could potentially be utilised by *Ulva* as a nitrogen source.

4) Sheltered urban sites. These were embayments or estuaries that were potentially impacted by urban nutrient sources. They were represented by sites near urban centres and included one site associated with *Ulva* blooms (see below).

5) Exposed or semi-exposed urban sites. These were either on open coasts or in large harbours with a moderate fetch and were potentially influenced by urban nutrient sources.

6) Enriched urban sites. These sites were proximal to known discharge sources and therefore should, on average, have had high nutrient loading. With the exception of one site at the Saint Kilda beach discharge point in Dunedin, all urban enriched sites were classified as sheltered. Exposed enriched urban sites were not specifically included as a category because coastal discharge pipes typically can

extend some distance (and depth) out to sea (e.g., Moa Point discharge), therefore it was considered not practical to sample these sites at the point of discharge.

All categories (with the exception of rock pools) were deemed sub-tidal since they were covered with about 200 mm of seawater at the time of sampling at low tide (note that since cotidal lines move around New Zealand in a clockwise direction it is possible to follow midday low tides on roughly consecutive days by travelling clockwise around the country). In addition, while no salinity measurements were made every effort was made to use sites that were predominantly marine. For example, in sheltered estuaries sites were located as close as possible to mouths or entrances without affecting their 'sheltered' status. In the case of sites on exposed coasts locations near large rivers were avoided.

Categorisation of sheltered or exposed sites was based on general observations (including fetch and open sea angle and substrate type), and for sheltered sites, the likelihood of anything more than small, wind-driven surface waves. Included within these categories were sites associated with two well-studied bloom-forming *Ulva* populations. These were located at Tauranga Harbour (represented in both seasons by a site at Otumoetai (OT)) and at the Avon-Heathcote estuary, Christchurch (represented in both seasons at Ebbtide Road (ET)) (Figure 2.1 and Table 2.1). Note that the Otumoetai site did not have any nearby effluent discharges and was therefore classified as sheltered urban. On the other hand, the Avon-Heathcote Estuary has been documented to be enriched with nutrients from two sewage treatment plants discharging a total of about 4.6 tonnes of dissolved nitrogen per day into the estuary around the time of sampling (Christchurch City Council, 2003).

In addition to the summer and winter surveys, one site at Napier was monitored (on a monthly basis) for two years from the beginning of 2002 to the beginning of 2004. This was used to evaluate how well the choice of summer and winter sampling times (February and August) might represent seasonal extremes reflected in *Ulva* N-status.

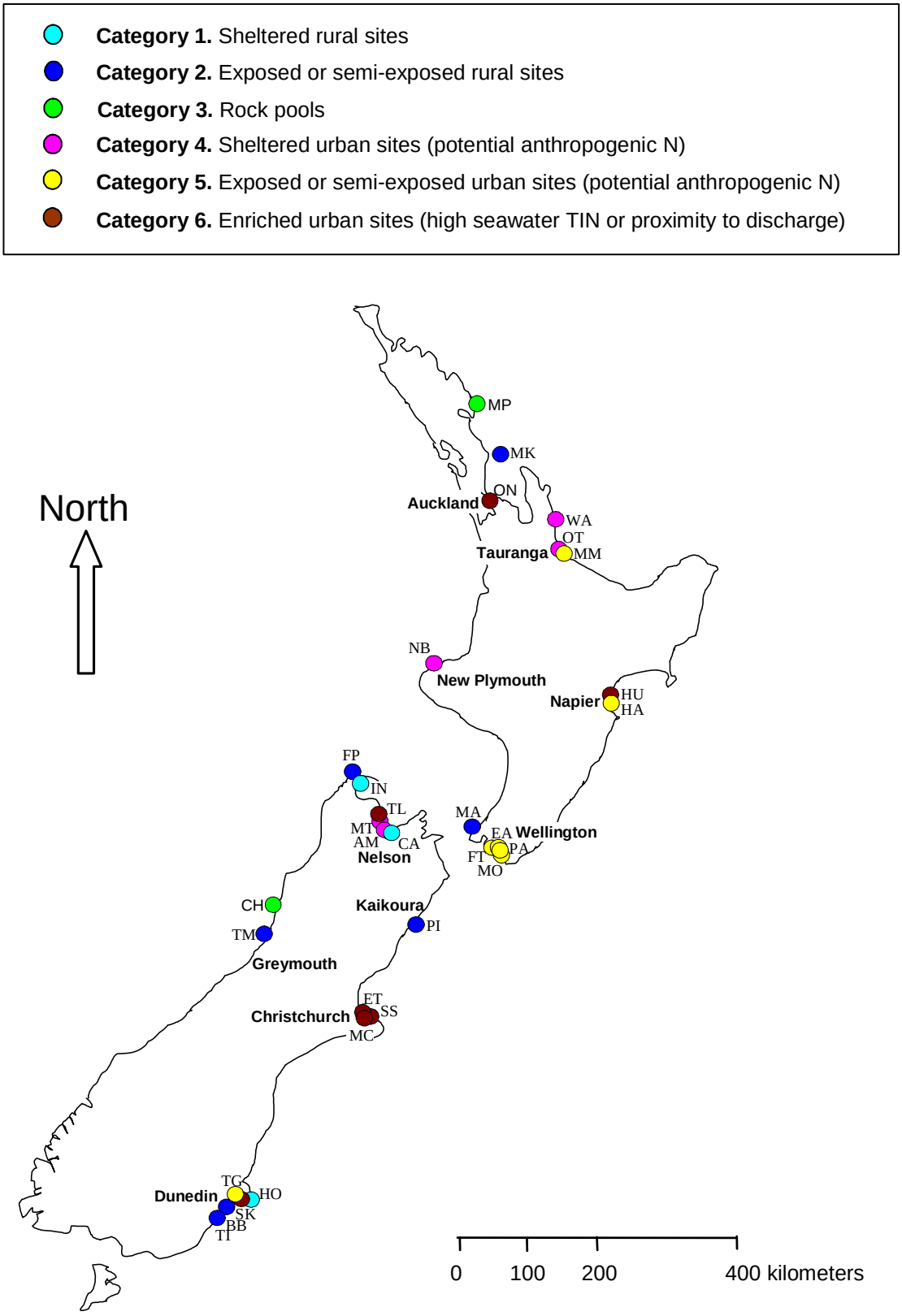



**Figure 2.1.** Location of sites in New Zealand from which *Ulva* was collected in the summer and winter of 2002.

**Table 2.1.** Sites and site descriptions for *Ulva* collections conducted in summer and winter 2002. Sites are ordered from north to south (see also Appendix 2.1).

| Code | Location | Region | North / South Is. | Site description | Substrate | Fetch | Exposure | Urban / rural | Summer | Winter |
|---|---|---|---|---|---|---|---|---|---|---|
| **MP** | Mermaid Pools | Northland | North | high rock pool | rock | moderate | sheltered | rural | √ | √ |
| **MK** | Mokohinau Islands | Northland | North | offshore island | rock | long | exposed | rural | √ | √ |
| **ON** | Onehunga lagoon | Auckland, west coast | North | sheltered tidal pond | shell / mud / free | short, very | sheltered | urban | √ | √ |
| **WA** | Whangamata Estuary | Whangamata | North | sheltered harbour | shell / sand | short | sheltered | urban | √ | √ |
| **MM** | Mount Manganui | Tauranga | North | moderatelty sheltered bay | rock | moderate | exposed | urban | √ | × |
| **OT** | Otumoetai | Tauranga | North | large harbour | shell / sand | moderate | exposed | urban | √ | √ |
| **HA** | Hardinge Road | Napier | North | sheltered embayment | rock / gravel | moderate | exposed | urban | √ | √ |
| **HU** | Humber Road drain | Napier | North | sheltered embayment | shell / mud | short, very | sheltered | urban | √ | × |
| **NB** | Ngamutu Beach | New Plymouth | North | small harbour | gravel / sand | short | sheltered | urban | √ | √ |
| **MA** | Makara Bay | Wellington | North | moderatelty sheltered bay | rock | long | exposed | rural | √ | √ |
| **FT** | Ferry Terminal | Wellington | North | large harbour | rock | moderate | exposed | urban | √ | √ |
| **EA** | Days Bay | Wellington | North | large harbour | rock / gravel | moderate | exposed | urban | √ | √ |
| **PA** | Point Arthur | Wellington | North | large harbour | rock / gravel | moderate | exposed | urban | × | √ |
| **MO** | Moa Point discharge | Wellington | North | exposed embayment | rock | long | exposed | urban | √ | √ |
| **FP** | Fossil Point | Farewell Spit | South | exposed rocky coast | rock | long | exposed | rural | √ | √ |
| **FW** | Farewell Bridge | Farewell Spit | South | channel, strong tidal current | rock | short, very | sheltered | rural | × | √ |
| **IN** | Inner Beach | Farewell Spit | South | sheltered embayment | shell / sand | short | sheltered | rural | √ | √ |
| **CA** | Cable Bay | Nelson | South | sheltered embayment | shell / sand | short | sheltered | rural | √ | √ |
| **AM** | Amatel Wharf | Nelson | South | sheltered embayment | shell / sand | short | sheltered | urban | √ | √ |
| **CH** | Charlston rockpools, Constance Bay | West Coast | South | high rock pool | rock | moderate | sheltered | rural | √ | √ |
| **TL** | Talleys Factory discharge | Motueka | South | sheltered embayment | rock/silt | short | sheltered | urban | √ | √ |
| **MT** | Motere Inlet | Motueka | South | sheltered embayment | hash/silt | short | sheltered | urban | √ | √ |
| **TM** | Tirimoana, Fox River mouth | West Coast | South | exposed rocky coast | rock | long | exposed | rural | √ | √ |
| **PI** | Paia Point | Kaikoura | South | exposed rocky coast | rock | long, very | exposed | rural | √ | √ |
| **ET** | Ebbtide Road, Avon-Heathcote Estuary | Christchurch | South | sheltered estuary | hash / silt | short | sheltered | urban | √ | √ |
| **MC** | McCormacks Bay, Avon Heathcote Estuary | Christchurch | South | sheltered estuary | shell / mud | short, very | sheltered | urban | × | √ |
| **SS** | South Spit Road, Avon-Heathcote Estuary | Christchurch | South | sheltered estuary | shell / sand | short | sheltered | urban | √ | × |
| **HO** | Hoopers Inlet | Dunedin | South | sheltered inlet | shell / mud | short | sheltered | rural | × | √ |
| **TG** | Taggart Road, Dunedin Harbour | Dunedin | South | large harbour | rock / gravel | moderate | exposed | urban | √ | √ |
| **SK** | Saint Kilda sewage discharge | Dunedin | South | exposed rocky coast | rock | long | exposed | urban | × | √ |
| **BB** | Brighton beach | Dunedin | South | exposed rocky coast | rock / sand | long | exposed | rural | √ | √ |
| **TI** | Taieri | Dunedin | South | exposed estuary mouth | rock | long | exposed | rural | √ | √ |

### 2.3.2 General weather conditions

Weather conditions for the summer survey were mostly fine at the beginning of the survey. However, the weather had deteriorated with more rainfall by early to mid February while visiting the South Island sites (Table 2.2). During the winter survey weather conditions were generally more settled and fine compared with the summer survey period.

While no site-specific meteorological data was available, data was obtained from the closest land weather stations (NIWA climate database, 2002), collated and presented as means for either North Island or South Island. The only gross comparisons in meteorological data that were not statistically significantly different were in total monthly rainfall values for the North Island between seasons, and for mean surface irradiance levels between the North and South Islands in both summer and winter (Table 2.2). There were two-fold differences in levels of surface irradiance between seasons in all comparisons with summer and winter values at around 20 MJ $\cdot$ $m^{-2}$ $\cdot$ $d^{-1}$ and 10 MJ $\cdot$ $m^{-2}$ $\cdot$ $d^{-1}$, respectively. There was more rainfall in the summer than in the winter for all sites and more rainfall in the summer in the South Island sites compared with those in the North Island. Conversely, in the winter there was more rainfall at the North Island sites compared with those of the South Island (Table 2.2).

**Table 2.2.** Comparison of meteorological data for one month preceding the summer and the winter surveys of *Ulva* in 2002. Analyses were conducted using Mann-Whitney rank sum tests for a difference.

| | Rainfall (mm) | | | Surface irradiance (MJ $\cdot$ $m^{-2}$ $\cdot$ $d^{-1}$) | | |
|---|---|---|---|---|---|---|
| | **Summer** | **Winter** | **Difference** | **Summer** | **Winter** | **Difference** |
| **All sites** | 110 ± 9 (n = 27) | 73 ± 6 (n = 29) | $P < 0.001$ | 21 ± 1 (n = 27) | 10 ± 0 (n = 29) | $P < 0.001$ |
| **North Island** | 88 ± 8 (n = 13) | 87 ± 5 (n = 12) | $P = 0.624$ | 22 ± 1 (n = 13) | 10 ± 1 (n = 12) | $P < 0.001$ |
| **South Island** | 130 ± 14 (n = 14) | 63 ± 9 (n = 17) | $P < 0.001$ | 20 ± 1 (n = 14) | 9 ± 0 (n = 17) | $P < 0.001$ |
| Difference | $P = 0.011$ | $P = 0.003$ | | $P = 0.482$ | $P = 0.313$ | |

***Algal sampling***

### 2.3.3 Algal taxonomy

Mainly frondose *Ulva* species were collected in this survey, but on some occasions *U. intestinalis* (syn. *Enteromorpha intestinalis*), being morphologically very distinct from the frondose form, was also collected for the purpose of biochemical comparison with other *Ulva* species. Several known species were sampled in this survey including: *U. pertusa*, *U. fasciata*, *U. scandinavica*, *U. spathulata* and *U. intestinalis*. While *Ulva* species like *U. intestinalis* and *U. spathulata* (Adams, 1994) were easily identified, others were not, since identification based on morphology alone is generally not reliable for this genus. Therefore it is probable that since not all samples could be positively identified other species may have been included. Some samples were identified from DNA sequencing (Maggs and McIvor, 2004; Heesch, 2006).

### 2.3.4 Sample standardisation

At each site, in summer and winter, three separate sampling areas were randomly selected within the main population beds (i.e. the largest or most obvious populations at each site were sampled). Haphazard selection was carried out in each area from individuals which met the following criteria:

- large thalli (150 – 250 mm)
- healthy and not showing signs of sporulation (i.e. fully green)
- attached to substrate
- covered with approximately 200mm seawater at low tide
- not shaded by topography or other algae

In rocky coastal areas, where algae tended to have a more patchy distribution, individuals within a reasonable (100 m) stretch of shore were sampled where patches were most dense. For the purposes of standardisation all algae and seawater samples were collected as close as possible to midday to minimise the effects of diurnal cycle and photoperiod.

***Thalli sampling.*** An investigation was made to evaluate if any biochemical gradients occurred in *Ulva* thalli as this would potentially confound sampling (i.e., values obtained might depend more on which part of the thallus was sampled). Three *Ulva pertusa* individuals were collected from the Mokohinau Islands (35° 54' 27.29 S, 175° 6' 25.99 E) and each one was divided into three approximately equal (by area) sections: proximal (including the holdfast and rhizoidal region), median and distal. Within each of these sections several 20 mm discs were randomly sampled using a leaf corer and determined for chlorophyll content. Chlorophyll was used as a proxy for nitrogen content (Barr and Rees, 2003). There was a decrease in total chlorophyll content (TChl) towards the distal end of the thallus (Figure 2.2).

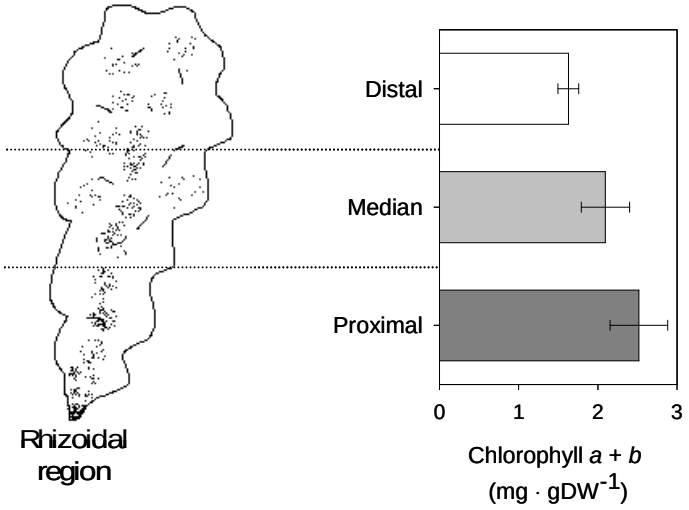



**Figure 2.2.** Variation in levels of chlorophyll *a* + *b* in *Ulva pertusa* thalli

Since there was potential for biochemical gradients to occur within *Ulva* thalli a method was developed for sub-sampling homogenised individual thalli (since this made no assumptions about where and when biochemical gradients occur within the thalli). After patting dry with paper tissues, *Ulva* thalli were cut into small (10 – 20 mm) pieces with scissors and then mixed by loosely tossing (like a salad). This proved to be a much quicker method than either randomly removing portions by hand or with a leaf corer. After cutting, fragments were then randomly sub-sampled to make up the required weight for biochemical analysis.

***Large, frondose* Ulva.** Three replicate whole thalli were surface dried with paper towels and sub-sampled as described above. Samples were then either quickly weighed and extracted for amino acids, or stored by freezing in small (50 × 70 mm) labelled zip lock bags for later analyses of tissue nitrogen and chlorophyll content. In the winter many *Ulva* individuals tended to be smaller (< 4 g) than in summer, and in some cases it was necessary to use composite sampling to ensure that there was enough tissue to do all the analyses. In these cases composite samples consisted of three thalli per replicate, which were sub-sampled and processed as described. Latex gloves, first rinsed in seawater, were worn before handling samples to minimise contamination.

**Ulva intestinalis.** Three replicate portions of tissue were collected and surface dried using paper towels and the treated as described above for frondose *Ulva*.

### 2.3.5 Algal tissue storage and integrity

Tissue and other samples were stored for the duration of the trip (up to 1 month for both surveys) at -26 ˚C in a purpose-built, fridge / freezer unit (Plate 2.1) supplied

**Plate 2.1**. Portable fridge / freezer unit for storage of samples

from a vehicle battery. Since there was the possibility of sample degradation through thawing, three systems were used to check the integrity of frozen samples. Firstly, the unit was equipped with a high-temperature audible alarm with latching LED indication. Secondly, plastic test tubes were prepared before each trip with alternating layers of frozen water and food-coloured water which were kept in the freezer along with samples for the duration of the trip. From these it was deduced that the freezer had maintained samples in a frozen state for the entire time in the field (i.e., there was no evidence of mixing of the frozen coloured layers). Finally, amino acid standards, that were used later to measure the concentration of amino acids in the unknown samples, were kept in the sample storage freezer for the duration of each trip. These standards were also compared with standards maintained continuously at -80 ˚C.

***Analytical***

### 2.3.6 Amino acid extraction

Amino acid samples were extracted from 1 g fresh weight *Ulva* tissue as described in Barr and Rees (2003) with the following modifications. Amino acids were extracted with 5 ml cold (4 ˚C) 1M perchloric acid for 10 min, before neutralising with 1M KOH/0.2 M MOPS. After 60 min at 4 ˚C approximately 1 ml supernatant was drawn off (avoiding the perchlorate salt precipitate), and placed into labelled 1.5 ml microcentrifuge tubes and stored frozen. The extracted tissue was squeezed dry between paper towels to remove most of the remaining liquid and stored frozen in labeled zip-lock bags for later determination of dry weight. Dry weight was determined by first rinsing with distilled water and then drying to a constant weight at 65 ˚C. Values of amino acids were quantified as described by Barr and Rees (2003) by comparison with external combination standards (see also Appendix 2.2).

Tryptophan, cystine and cysteine were not present in detectable amounts (Lourenco *et al.*, 2002). Glycine and citrulline co-eluted using our method and while attempts were made to separate these two amino acids, this resulted in poorer separation of other amino acids, particularly aspartate and glutamate. Given that glycine and citrulline were always present in low yields (around 1 % of the total free amino acid pool), and that amino acids glutamate and aspartate were more important in terms of nitrogen metabolism, values of glycine and citrulline are expressed together as an assumed equal contribution of N (i.e., 2 N since glycine has 1N and citrulline has 3N).

There were two amino-containing compounds that were regularly detected in *Ulva* samples. The first of these had a slightly later elution time than histidine (which eluted at 8.40 minutes) using the method of Barr and Rees (2003). However, this unknown was typically present in small amounts and was only problematic with the *Ulva* species examined in Chapter Four. Conversely, the second of the unknowns was often present in large amounts. There was insufficient data to conclusively resolve its identity using both mass spectrometry and microanalysis (Wilcox, 2004). It had an atomic mass of 248 with 3.1 % nitrogen, 21 % carbon (by NMR 5 or 7 carbons) and 4.3 % hydrogen. It was possibly an amino betaine compound (Wilcox, 2004). When referred to, this compound is denoted as $U2^{9.48}$ since it eluted at 9.48 minutes using the method described above (Appendix 2.3).

### 2.3.7 Chlorophyll

Chlorophyll was extracted after thawing from 0.3 g fresh weight of *Ulva* tissue sample in 15 ml methanol / DMSO (4 : 1 v/v) as described by Duncan and Harrison (1982) for 24 hours at 4 °C. Absorbances were measured at three wavelengths and converted to chlorophyll concentrations using the following formulae (Holden, 1965).

$$\text{Chlorophyll a } (\mu g \cdot mL^{-1}) = (16.5 \times (Abs^{665} - Abs^{750})) - (8.3 \times (Abs^{650} - Abs^{750}))$$

$$\text{Chlorophyll b } (\mu g \cdot mL^{-1}) = (33.8 \times (Abs^{650} - Abs^{750})) - (12.5 \times (Abs^{665} - Abs^{750}))$$

Values are expressed as $mg \cdot g\ DW^{-1}$.

### 2.3.8 Tissue nitrogen content

After thawing and washing with distilled water, 1 g fresh weight of *Ulva* tissue sample was dried at 65 °C for 24 hours. After grinding, nitrogen content and nitrogen isotopes ($\delta^{15}N$) were analysed at the Waikato Isotope Unit, University of Waikato. A Dumas elemental analyser (Europa Scientific ANCA-SL) interfaced to a isotope mass spectrometer (Europa Scientific 20-20 Stable Isotope Analyser) was used. Samples were analysed against a urea standard / reference with a delta value of -0.45 ‰.

### 2.3.9 Seawater sampling and nutrient analyses

Seawater samples were taken at the same time and place as seaweed samples. Seawater temperatures were recorded, using a thermometer, at the same time as seawater samples were taken. Samples were kept chilled at 4 ˚C and analysed within 12 hours of collection. These were analysed for ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$) and phosphate ($PO_4^{3-}$) using a HACH DR2000 portable spectrophotometer. Ammonium was determined as described by Koroleff (1983a) and nitrite by Parsons *et al* (1984). Nitrate was reduced to nitrite using cadmium pillows (supplied by HACH, product code: NitraVer 6), and then determined using the nitrite method described above. Phosphate was determined as described by Koroleff (1983b). Total inorganic nitrogen (TIN) concentrations were calculated as the sum of $NH_4^+$, $NO_2^-$ and $NO_3^-$. The ratio of nitrogen : phosphorus (N : P) was calculated as TIN : $PO_4^{3-}$. Concentrations values are expressed in μM.

### 2.3.10 Statistical analysis

Regression lines were fitted by ordinary least squares in SigmaStat 3.10. Means were compared using a two-way or three-way general linear model (analyses of variance). The Holm-Sidak method was used to compare among means. All data analysed met assumptions of normality or equal variance. Non-parametric ordination of *Ulva* N-indices was conducted with the statistical software package PRIMER (Version 5) (Clarke and Gorley, 2001).

## 2.4 Results

### 2.4.1 Summer and winter seawater temperature

Seawater temperatures were variable but showed a general decline with increasing latitude, in both summer and winter (Figure 2.3). Seawater temperatures in the summer ranged from 15.5 ˚C at Taieri River mouth to 27 ˚C at Motere Inlet in Motueka. The average for all sites in the summer was 20.1 ± 0.6 ˚C (Table 2.3). During the winter, seawater temperatures ranged from 9.8 ˚C at Paia Point, Kaikoura

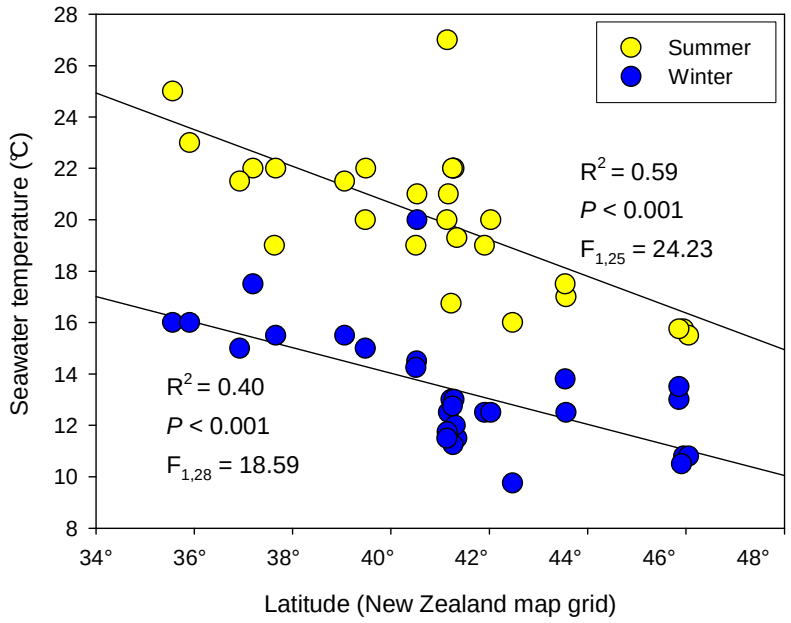



**Figure 2.3.** Change in seawater temperature with change in latitude for summer and winter 2002.

**Table 2.3.** Seawater temperatures pooled for each site category for summer and winter 2002.

| | Sheltered rural | Exposed rural | Rock pools | Sheltered urban | Exposed urban | Enriched urban | Mean |
|---|---|---|---|---|---|---|---|
| | (Seawater temperature in °C) | | | | | | |
| **Summer** | 21.0 ± 0.0 (n = 2) | 18.0 ± 1.1 (n = 7) | 22.0 ± 3.0 (n = 2) | 22.9 ± 1.0 (n = 5) | 19.7 ± 0.9 (n = 6) | 19.6 ± 1.0 (n = 5) | 20.1 ± 0.6 (n = 27) |
| **Winter** | 15.2 ± 2.4 (n = 3) | 12.4 ± 0.8 (n = 8) | 14.3 ± 1.8 (n = 2) | 14.6 ± 1.0 (n = 5) | 12.7 ± 0.6 (n = 6) | 12.7 ± 0.8 (n = 5) | 13.4 ± 0.4 (n = 29) |

to 20 ˚C at Inner Beach, Farewell Spit (Figure 2.3). The winter average for all sites was 13.4 ± 0.4 ˚C. Mean category temperatures were similar between categories in both summer and winter surveys. However, exposed coastal sites had slightly lower mean temperatures than other categories in both seasons (Table 2.3).

### 2.4.2 Long-term (2002 – 2004) monitoring of chlorophyll levels in *Ulva fasciata*

Chlorophyll levels in *Ulva fasciata* were monitored over two years at Hardinge Road, Napier (HA). Although peaks in chlorophyll values were different between the winters of 2002 and 2003, summer values were lowest from December to February (Figure 2.4). From these results it is suggested that the choice of summer and winter sampling times was likely to reflect seasonal extremes in *Ulva* N-status.

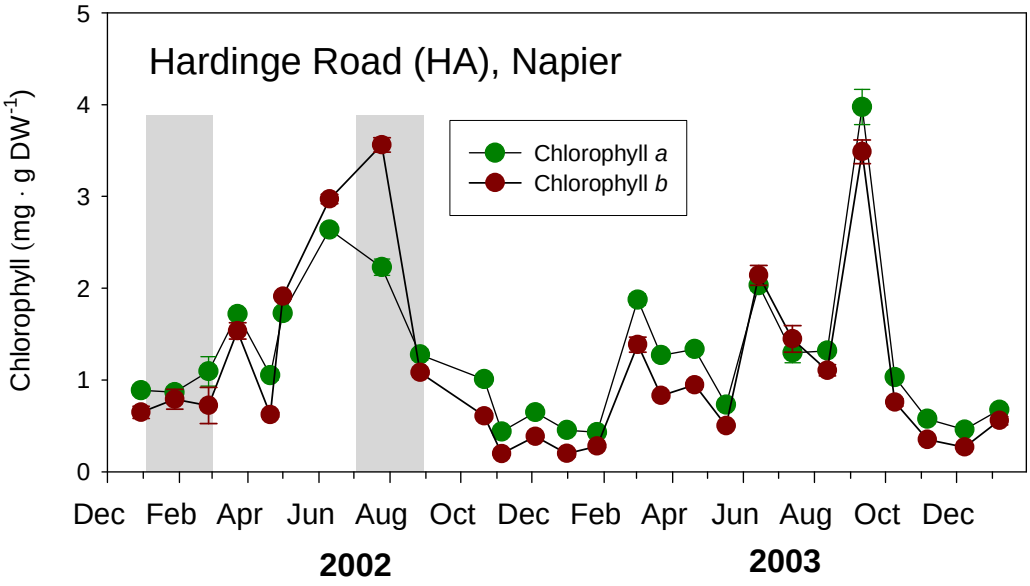



**Figure 2.4.** Change in chlorophyll content in *Ulva* at a site in Hardinge Road, Napier, from 2002 to 2003. Shaded bars indicate when most sampling was conducted around New Zealand.

### 2.4.2 Summer seawater nutrients and *Ulva* N-indices

***Seawater nutrients***

Measured seawater nutrients were highly variable between sites. Total inorganic nitrogen (TIN) concentrations ranged from close to detectable limits (0.1 ± 0.0 µM at Hardinge Road (HA, exposed urban), Napier, to 109.2 ± 2.2 µM at Ebbtide Road (ET, enriched urban) in the Avon-Heathcote estuary, Christchurch (Appendix 2.4).

The category with the highest mean TIN concentration was the enriched urban sites with a mean value of 38.2 ± 19.3 µM. The lowest TIN concentrations were measured at the exposed urban sites (1.3 ± 0.7 µM) and the sheltered rural sites (2.2 ± 1.7 µM) (Table 2.4).

Ammonium ($NH_4^+$) was the dominant inorganic nitrogen source for most categories, particularly the enriched urban (75.9 ± 17.7 %) and rock-pool (89.3 ± 9.5 %) sites (Table 2.4). Enriched urban sites had the highest mean concentration of phosphorus ($PO_4^{3-}$) at 7.0 ± 3.9 µM while the rock-pool sites had the lowest at 0.2 ± 0.1 µM (Table 2.4). The category with the highest nitrogen : phosphorus ratio (N : P) was the rock pool sites (42.6 ± 7.4) while sheltered rural sites had the lowest (4.2 ± 3.4) (Table 2.4).

**Table 2.4.** Seawater nutrient concentrations pooled for each category for summer 2002. Minimum values for category means are indicated in blue text and the maximum values are indicated in red text.

| | Sheltered rural | | Exposed rural | | Rock pools | | Sheltered urban | | Exposed urban | | Enriched urban | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n = 2 | | n = 7 | | n = 2 | | n = 5 | | n = 6 | | n = 5 | |
| **Seawater nutrients** | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| **TIN** (µM) | 2.2 | 2.3 | 4.0 | 4.9 | 7.5 | 5.7 | 4.3 | 2.6 | **1.3** | 1.7 | **38.2** | 43.1 |
| $NH_4^+$ (µM) | 2.0 | 2.5 | 2.1 | 2.0 | 7.1 | 6.1 | 2.4 | 1.2 | **1.0** | 1.6 | **30.8** | 36.7 |
| $NO_2^-$ (µM) | 0.1 | 0.0 | 0.2 | 0.1 | **0.0** | 0.0 | 0.2 | 0.1 | 0.1 | 0.1 | **1.2** | 1.1 |
| $NO_3^-$ (µM) | **0.2** | 0.1 | 1.8 | 3.8 | 0.4 | 0.4 | 1.7 | 2.1 | 0.3 | 0.2 | **6.2** | 6.0 |
| $NH_4^+$ / TIN (%) | 69.6 | 38.2 | 63.7 | 28.8 | **89.3** | 13.4 | 63.8 | 22.2 | **44.4** | 36.2 | 75.9 | 17.7 |
| **$PO_4^{3-}$** (µM) | 0.6 | 0.1 | 0.6 | 0.5 | **0.2** | 0.1 | 0.5 | 0.3 | 0.5 | 0.3 | **7.0** | 8.7 |
| N : P | 4.2 | 4.9 | 5.3 | 2.9 | **42.6** | 10.5 | 11.1 | 9.5 | **2.9** | 3.9 | 12.2 | 16.0 |

### *Nitrogen indices in* Ulva

In the summer the greatest difference between any two categories was seen in sheltered rural and enriched urban sites (Table 2.5). In all cases *Ulva* N-indices were the highest in the enriched urban sites and in most cases the lowest in the sheltered rural sites (Table 2.5). Moreover, *Ulva* from enriched sites had higher levels of free amino acids (total and individual), chlorophyll a, chlorophyll b, total chlorophyll

(TChl) and total tissue nitrogen (TN) compared with any other category (see also Appendix 2.5).

The free amino acid (FAA) N-indices generally showed the largest differences across the range of categories compared with chlorophyll and TN values. For example, both the total FAA pool and asparagine (Asn) showed the largest range across the categories (19.8 ± 5.2 to 173.7 ± 14.5 µmol N · g $DW^{-1}$ and 0.9 ± 0.0 to 93.2 ± 9.2 µmol N · g $DW^{-1}$, respectively) (Table 2.5). Compared to asparagine, levels of glutamine (Gln), glutamate (Glu) and aspartate (Asp) were present in relatively low (< 10 µmol N · g $DW^{-1}$) values across all categories. Gln : Glu showed a small range (0.2 to 0.6) across the categories (Table 2.5). Levels of histidine (His) were lower in sheltered rural sites than other categories, and similar (22.8 to 25.1 µmol N · g $DW^{-1}$) in exposed rural, and sheltered and exposed urban sites (Table 2.5). Mean levels of

**Table 2.5.** Nitrogen indices in *Ulva* pooled for each category for summer 2002. Minimum values for category means are indicated in blue text and the maximum values are indicated in red text.

| | Sheltered rural | | Exposed rural | | Rock pools | | Sheltered urban | | Exposed urban | | Enriched urban | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n = 2 | | n = 7 | | n = 2 | | n = 5 | | n = 6 | | n = 5 | |
| **Nitrogen indices in *Ulva*** | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| **total FAA** (µmol N · g $DW^{-1}$) | **19.8** | 7.4 | 46.5 | 15.7 | 46.5 | 1.4 | 64.4 | 32.9 | 61.8 | 63.1 | **173.7** | 32.5 |
| Asn (µmol N · g $DW^{-1}$) | 1.5 | 1.7 | 8.7 | 12.0 | **0.9** | 0.0 | 18.4 | 19.0 | 13.8 | 20.5 | **93.2** | 20.5 |
| Asp (µmol N · g $DW^{-1}$) | **0.5** | 0.0 | 0.9 | 0.2 | 1.1 | 0.2 | 1.3 | 0.6 | 1.2 | 0.9 | **2.5** | 0.4 |
| Asn : Asp | 3.1 | 3.4 | 8.6 | 11.1 | **0.8** | 0.1 | 13.0 | 12.0 | 8.7 | 8.5 | **37.0** | 8.9 |
| Gln (µmol N · g $DW^{-1}$) | **1.3** | 0.1 | 2.1 | 1.6 | 4.1 | 0.8 | 2.4 | 1.5 | 2.5 | 1.3 | **7.7** | 5.6 |
| Glu (µmol N · g $DW^{-1}$) | **2.3** | 0.1 | 2.8 | 1.4 | 3.8 | 0.7 | 5.2 | 2.6 | 4.4 | 3.2 | **6.3** | 3.7 |
| Gln : Glu | 0.3 | 0.0 | 0.4 | 0.2 | 0.5 | 0.0 | **0.2** | 0.1 | 0.4 | 0.2 | **0.6** | 0.3 |
| His (µmol N · g $DW^{-1}$) | **7.2** | 2.9 | 22.8 | 6.7 | 25.1 | 1.3 | 24.8 | 10.9 | 24.6 | 23.7 | **35.4** | 6.1 |
| Pro (µmol N · g $DW^{-1}$) | **0.8** | 0.7 | 2.2 | 1.3 | 2.1 | 0.8 | 3.7 | 3.7 | 5.2 | 7.1 | **10.7** | 11.4 |
| $U2^{9.48}$ (µmol N · g $DW^{-1}$) | **0.7** | 0.2 | 1.1 | 0.5 | 2.0 | 0.5 | 1.4 | 0.8 | 3.2 | 6.2 | **6.4** | 3.3 |
| Others (µmol N · g $DW^{-1}$) | **5.5** | 1.8 | 6.0 | 1.0 | 7.5 | 2.0 | 7.1 | 2.2 | 6.9 | 2.0 | **11.5** | 4.1 |
| chlorophyll *a* (µg · g $DW^{-1}$) | **0.9** | 0.3 | 2.2 | 0.8 | 2.8 | 0.1 | 2.0 | 1.1 | 1.7 | 0.9 | **3.1** | 0.5 |
| chlorophyll *b* (µg · g $DW^{-1}$) | **0.6** | 0.2 | 1.7 | 0.6 | 1.7 | 0.1 | 1.7 | 0.9 | 1.4 | 0.6 | **4.2** | 3.1 |
| chlorophyll *a* + *b* (µg · g $DW^{-1}$) | **1.5** | 0.5 | 3.9 | 1.3 | 4.5 | 0.3 | 3.7 | 2.0 | 3.1 | 1.5 | **7.3** | 3.4 |
| free amino N : total N (%) | **2.9** | 0.2 | 3.0 | 0.9 | 3.3 | 0.4 | 5.0 | 1.6 | 5.0 | 3.8 | **8.0** | 1.7 |
| total tissue N (%) | **1.0** | 0.3 | 2.3 | 0.4 | 2.0 | 0.2 | 1.7 | 0.5 | 1.6 | 0.6 | **3.1** | 0.5 |
| $\delta^{15}N$ (‰) | 7.5 | 0.4 | 7.8 | 0.6 | **7.0** | 0.5 | 8.3 | 1.0 | 8.7 | 1.3 | **10.2** | 3.5 |

proline (Pro) and U2[9.48], while low (< 10 µmol N · g $DW^{-1}$), did show a reasonable range of values across the categories. The remaining (other) amino acids, were also low (< 11.5 µmol N · g $DW^{-1}$) and varied less across the categories (Table 2.5).

The plot shown below (Figure 2.5) comparing FAA content in *Ulva* with stable nitrogen isotope ($\delta^{15}N$) content, indicates a separation of enriched urban sites (brown symbols) from most of the other sites in New Zealand. Values of $\delta^{15}N$ in *Ulva* showed the largest range at enriched urban sites compared with any other category. The lowest value of 6.0 ± 0.1 ‰ was recorded at Talley's fish factory discharge (TL) and the highest of 15.1 ± 0.1 ‰ at Onehunga lagoon (ON), Auckland. All exposed rural sites, representing clean coastal seawater, fell within a range of 6.7 ‰ to 8.8 ‰. Four of the five enriched urban sites (including ET) and three urban sites fell outside this range (Figure 2.5).

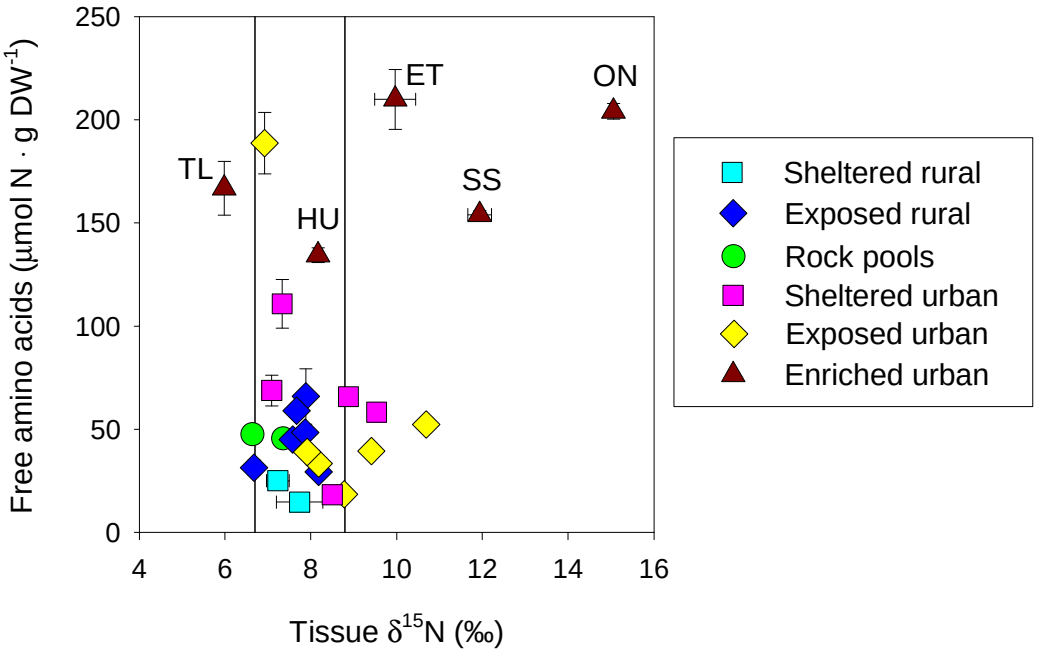



**Figure 2.5.** Comparison of free amino acid content with tissue $\delta^{15}N$ content in *Ulva* from sites around New Zealand in summer 2002. Range of $\delta^{15}N$ values for *Ulva* from exposed rural sites (6.7 ‰ to 8.8 ‰ ) indicated by vertical lines.

### 2.4.3 Winter seawater nutrients and *Ulva* N-indices

***Seawater nutrients***

As in summer, seawater nutrients in the winter were variable (Appendix 2.6). TIN concentrations ranged from 0.1 ± 0.1 µM at Tirimoana (TM, exposed rural), to 29.9 ± 0.3 µM at Ebbtide Road (ET, enriched urban) (Appendix 2.6). Similarly to the summer survey, the mean TIN concentration was highest (17.8 ± 4.3 µM) at the enriched urban sites (Table 2.6). The dominant source of TIN was $NH_4^+$ for three out of six categories, although exposed rural sites had similar $NO_3^-$ concentrations compared with the summer (Table 2.6). Mean $NH_4^+$ concentrations were lower compared to summer across all sites. In the winter (and in the survey overall) the highest level of nitrate was 19.7 ± 0.9 µM recorded at Makara Beach (MA, exposed rural) Wellington (Appendix 2.6).

**Table 2.6.** Seawater nutrients concentrations pooled for each category for winter 2002. Minimum values for category means are indicated in blue text and the maximum values are indicated in red text.

| | Sheltered rural | | Exposed rural | | Rock pools | | Sheltered urban | | Exposed urban | | Enriched urban | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n = 2 | | n = 8 | | n = 2 | | n = 5 | | n = 6 | | n = 5 | |
| **Seawater nutrients** | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| **TIN** (µM) | **0.7** | 0.3 | 3.7 | 6.6 | 1.5 | 0.8 | 1.9 | 0.9 | 1.4 | 0.8 | **17.8** | 9.5 |
| $NH_4^+$ (µM) | 0.6 | 0.3 | **0.5** | 0.2 | 1.1 | 0.5 | 0.8 | 0.5 | 0.6 | 0.7 | **13.0** | 8.3 |
| $NO_2^-$ (µM) | **0.1** | 0.1 | 0.2 | 0.2 | 0.2 | 0.0 | 0.2 | 0.1 | 0.2 | 0.1 | **0.5** | 0.4 |
| $NO_3^-$ (µM) | **0.1** | 0.1 | 3.0 | 6.4 | 0.2 | 0.3 | 0.9 | 0.6 | 0.6 | 0.7 | **4.4** | 3.8 |
| $NH_4^+$ / TIN (%) | 79.4 | 12.2 | **35.9** | 32.6 | 73.7 | 3.4 | 45.4 | 20.5 | 39.1 | 28.8 | **72.4** | 24.3 |
| **$PO_4^{3-}$** (µM) | 0.5 | 0.1 | 0.3 | 0.3 | **0.3** | 0.1 | 0.4 | 0.1 | 0.4 | 0.2 | **3.3** | 2.3 |
| N : P | **1.7** | 0.8 | **21.7** | 33.8 | 5.4 | 0.0 | 4.4 | 1.8 | 3.8 | 1.9 | 7.7 | 4.5 |

As in summer, the highest concentrations of $PO_4^{3-}$ were found in the enriched urban sites (Table 2.6). The nitrogen : phosphorus (N : P) ratio ranged from 0.9 ± 0.5 at Tirimoana (TM, exposed rural) on the South Island's west coast, to 92.8 ± 12.4 at Makara Beach (Appendix 2.6). Exposed rural coastal sites had the highest N : P ratio at 21.7 ± 11.9 while the sheltered rural sites (as in the summer) had the lowest at 1.7 ± 0.5 (Table 2.6).

### *Nitrogen indices in* Ulva

In the winter it was very apparent that N-indices in *Ulva* from all categories had increased relative to values found in summer (Table 2.7). While all N-indices in *Ulva* from sheltered rural sites were still clearly lower than those from enriched urban sites, the other categories of exposed rural, rock pools, sheltered urban and exposed urban showed winter increases in mean values often approaching and sometimes exceeding values seen at enriched urban sites (Table 2.7).

**Table 2.7.** Nitrogen indices in *Ulva* pooled for each category for winter 2002. Minimum values for category means are indicated in blue text and the maximum values are indicated in red text.

| | Sheltered rural | | Exposed rural | | Rock pools | | Sheltered urban | | Exposed urban | | Enriched urban | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n = 3 | | n = 8 | | n = 2 | | n = 5 | | n = 6 | | n = 5 | |
| **Nitrogen indices in *Ulva*** | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| **total FAA** (μmol N · g DW$^{-1}$) | **58.5** | 8.9 | 168.3 | 88.5 | 97.5 | 12.6 | 144.9 | 63.7 | 200.7 | 106.8 | **225.0** | 87.8 |
| Asn (μmol N · g DW$^{-1}$) | 9.9 | 1.9 | 77.7 | 53.6 | **5.6** | 5.5 | 81.9 | 41.1 | 86.0 | 51.4 | **107.5** | 49.2 |
| Asp (μmol N · g DW$^{-1}$) | **0.8** | 0.3 | 1.9 | 1.0 | 1.0 | 0.0 | 1.7 | 0.6 | 1.8 | 0.8 | **2.4** | 1.0 |
| Asn : Asp | 12.6 | 4.0 | 37.7 | 18.5 | **5.5** | 5.2 | **46.7** | 12.4 | 42.4 | 22.6 | 46.4 | 13.5 |
| Gln (μmol N · g DW$^{-1}$) | **2.1** | 0.8 | 5.2 | 3.4 | **15.0** | 7.1 | 2.9 | 1.1 | 3.5 | 1.4 | 11.9 | 8.7 |
| Glu (μmol N · g DW$^{-1}$) | **7.2** | 2.1 | 11.0 | 5.5 | 8.1 | 0.2 | 9.0 | 3.4 | **14.9** | 8.0 | 14.6 | 6.4 |
| Gln : Glu | **0.2** | 0.1 | 0.3 | 0.2 | **0.9** | 0.5 | 0.2 | 0.1 | 0.2 | 0.1 | 0.4 | 0.2 |
| His (μmol N · g DW$^{-1}$) | **17.8** | 1.9 | **40.6** | 12.3 | 33.2 | 1.8 | 24.9 | 17.2 | 36.9 | 18.3 | 39.5 | 10.5 |
| Pro (μmol N · g DW$^{-1}$) | **11.7** | 8.0 | 19.8 | 15.3 | 16.8 | 5.7 | 13.9 | 10.2 | **42.5** | 36.9 | 21.8 | 14.2 |
| U2$^{9.48}$ (μmol N · g DW$^{-1}$) | **1.1** | 1.1 | 3.0 | 3.1 | 7.4 | 2.8 | 2.0 | 0.7 | 3.7 | 3.1 | **8.6** | 8.9 |
| Others (μmol N · g DW$^{-1}$) | **8.0** | 0.6 | 9.0 | 3.7 | 10.4 | 3.6 | 8.7 | 3.5 | 11.5 | 6.0 | **18.8** | 11.7 |
| chlorophyll *a* (μg · g DW$^{-1}$) | **2.1** | 0.2 | 3.6 | 0.8 | 3.9 | 0.8 | 2.5 | 0.9 | 4.5 | 2.5 | **5.3** | 0.9 |
| chlorophyll *b* (μg · g DW$^{-1}$) | **1.5** | 0.1 | 2.8 | 0.9 | 2.4 | 0.4 | 2.0 | 0.7 | 2.9 | 1.1 | **4.5** | 1.5 |
| chlorophyll *a* + *b* (μg · g DW$^{-1}$) | **3.6** | 0.2 | 6.4 | 1.7 | 6.3 | 1.2 | 4.5 | 1.6 | 7.3 | 3.4 | **9.7** | 1.4 |
| free amino N : total N (%) | 4.6 | 0.4 | 6.6 | 2.9 | **4.5** | 0.9 | 8.3 | 1.6 | 7.6 | 2.5 | **8.4** | 2.7 |
| total tissue N (%) | **1.8** | 0.4 | 3.5 | 0.5 | 3.0 | 0.2 | 2.4 | 0.8 | 3.4 | 1.2 | **3.7** | 0.4 |
| $\delta^{15}$N (‰) | 7.7 | 1.4 | 7.6 | 0.6 | **7.5** | 0.7 | 7.7 | 0.5 | 8.6 | 0.7 | **8.8** | 3.9 |

Although less clear than the situation in summer, the combination of FAA content in *Ulva* with stable nitrogen isotope ($\delta^{15}$N) content (Figure 2.6) still showed a separation of sheltered rural sites (light blue symbols) and enriched urban sites (brown symbols). In the winter stable nitrogen isotopes ($\delta^{15}$N) in *Ulva* from enriched urban sites again had the largest range of values compared with other categories. The lowest value of

4.8 ± 0.0 ‰ was recorded at ET and the highest of 15.2 ± 0.0 ‰ at ON (Appendix 2.7). Exposed rural sites fell within the range of 6.7 ‰ to 8.5 ‰ (Figure 2.6 and Appendix 2.7). This was similar to values in the summer and again several sites, including ET and ON and two sheltered rural sites, fell outside this range (Figure 2.6).

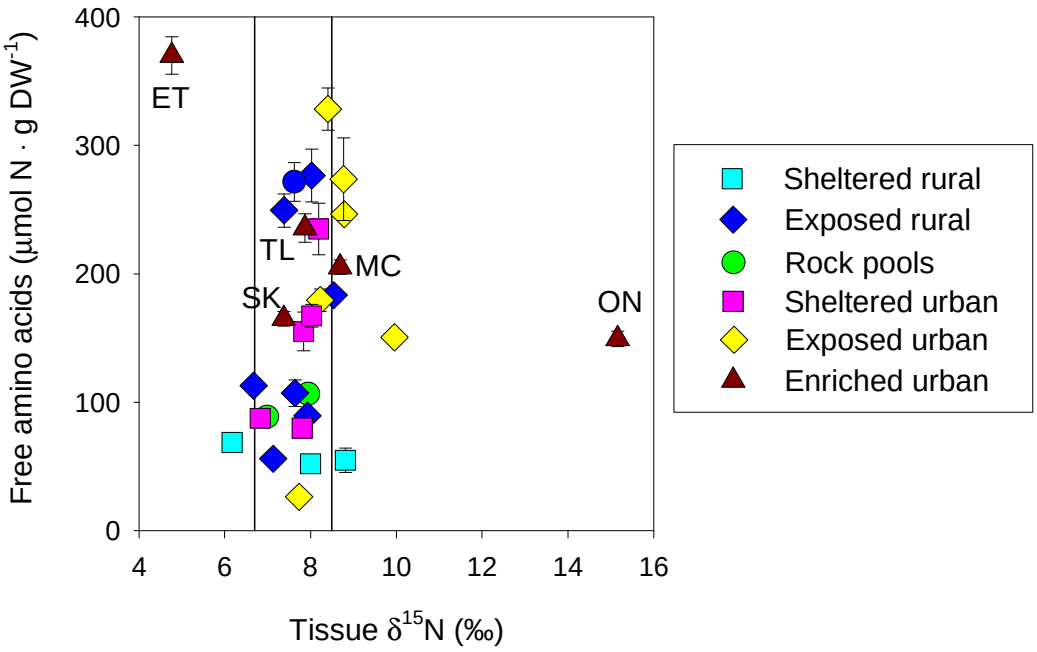



**Figure 2.6.** Comparison of free amino acid content with tissue $\delta^{15}N$ content in *Ulva* from sites around New Zealand in winter 2002. Range of $\delta^{15}N$ values for *Ulva* from exposed rural sites (6.7 ‰ to 8.5 ‰ ) indicated by vertical lines.

### 2.4.4 Ordination of *Ulva* N-indices using cluster analysis

Since simple correlative relationships between seawater nitrogen concentrations and *Ulva* N-indices were poor (Appendix 2.8 and 2.9), statistical ordination was used to explore how patterns in *Ulva* N-indices related to environmental variables. Ordination of selected N-indices was conducted with the statistical software package PRIMER (Version 5) (Clarke and Gorley, 2001). *Ulva* biochemical data were not constrained by external environmental factors so that only N-indices determined the similarity matrix relationships between the sites.

Similarity matrices were constructed for *Ulva* N-indices using normalised Euclidian distances for data from both seasons. This was done since indices had different units and often contained values less than 1 (Clarke and Warwick, 1994). Indices included: total free amino acids (FAA), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total tissue-N content. Environmental data were not included in the matrices; however seawater TIN concentration, seawater temperature and surface irradiance were compared with biochemical data by plotting histograms below plots for *Ulva* tissue-N indices. Seawater temperature and surface irradiance were presented as residuals based on overall means for each season (i.e. residual value = $\bar{x} - x$ ).

***Summer***

Cluster analysis of similarity between *Ulva* N-indices resulted in three distinctive groupings. Going from left to right in Figure 2.7, the three groups consisted of sites with *Ulva* containing low (A), medium (B), and high N-indices (C). The low tissue-N grouping (Figure 2.7, A) included the two sheltered rural sites (CA and IN), three exposed urban sites (HA, EA and TG), three sheltered urban site (WA, AM and OT) and one open coastal site (BB). The grouping containing *Ulva* with intermediate N-indices (Figure 2.7, B) included both rock-pool sites, most of the rural exposed sites and three urban sites (exposed and sheltered). All of the enriched urban sites were contained in one main group (C) with high N-indices (Figure 2.7, C). It is also worth noting that this group also had *Ulva* with distinctly higher levels of asparagine compared with the other two groups (Appendix 2.10). A notable sub-group within the high tissue-N group was *Ulva scandinavica* (Maggs and McIvor, 2004) from Onehunga lagoon (ON) which contained the highest levels of chlorophyll *b* recorded in the summer. In addition to the enriched urban sites in this group were one exposed urban site at Mount Maunganui (MM) and one sheltered urban site at Ngamutu Beach (NB); the latter having moderate TIN concentrations (6.51 ± 2.51 µM, Appendix 2.4) at the time *Ulva* was sampled. Although these sites were not adjacent to any known effluent source, both were adjacent to ports which handle fertiliser and suffer accidental spillages (Mundy, 2002; Trenwith, 2002) which might have been a factor explaining the higher N-indices in *Ulva* (and higher levels TIN in the case of Ngamutu Beach) at these sites. An alternative explanation of the high levels of N-indices (which weren't explained by elevated seawater TIN concentrations) in the sample from Mount Maunganui is that they represented some kind of genetic outlier

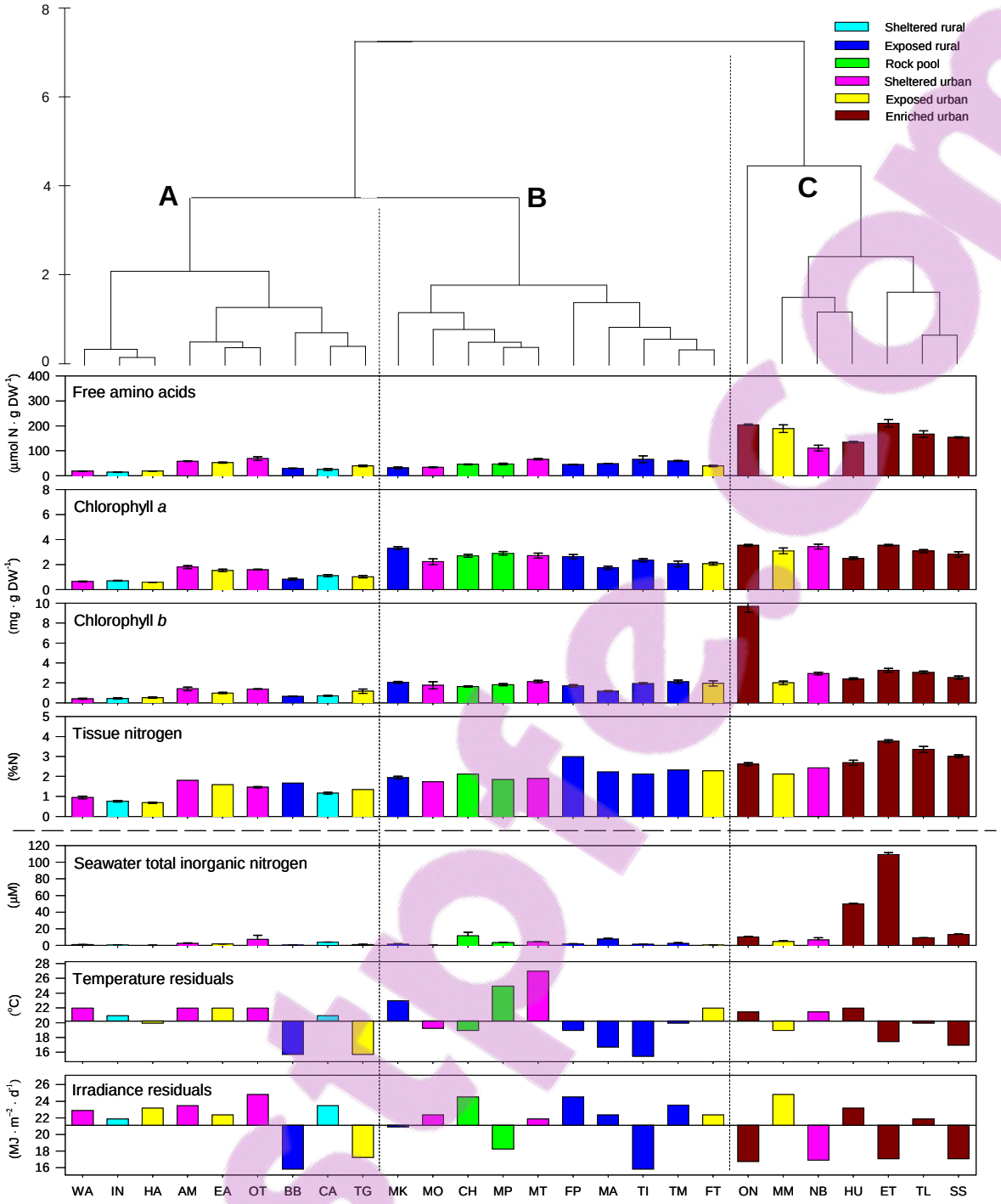



**Figure 2.7.** Similarity dendrogram plot of (normalised) Euclidian distances between five N-indices measured in 26 *Ulva* populations in summer 2002. Three main groupings (**A**, **B** and **C**) are separated by vertical dashed lines. Clustered groups of sites range from those with (**A**) *Ulva* containing low N-indices through an intermediate group of sites with (**B**) *Ulva* containing intermediate tissue-N indices, to a group of sites with (**C**) *Ulva* containing high tissue-N indices. Histograms of the five *Ulva* N-indices used to construct the dendrogram are shown below the cluster plot. Values for seawater total inorganic nitrogen concentration, and residual values calculated for seawater temperature and surface irradiance are shown in the bottom two histograms.

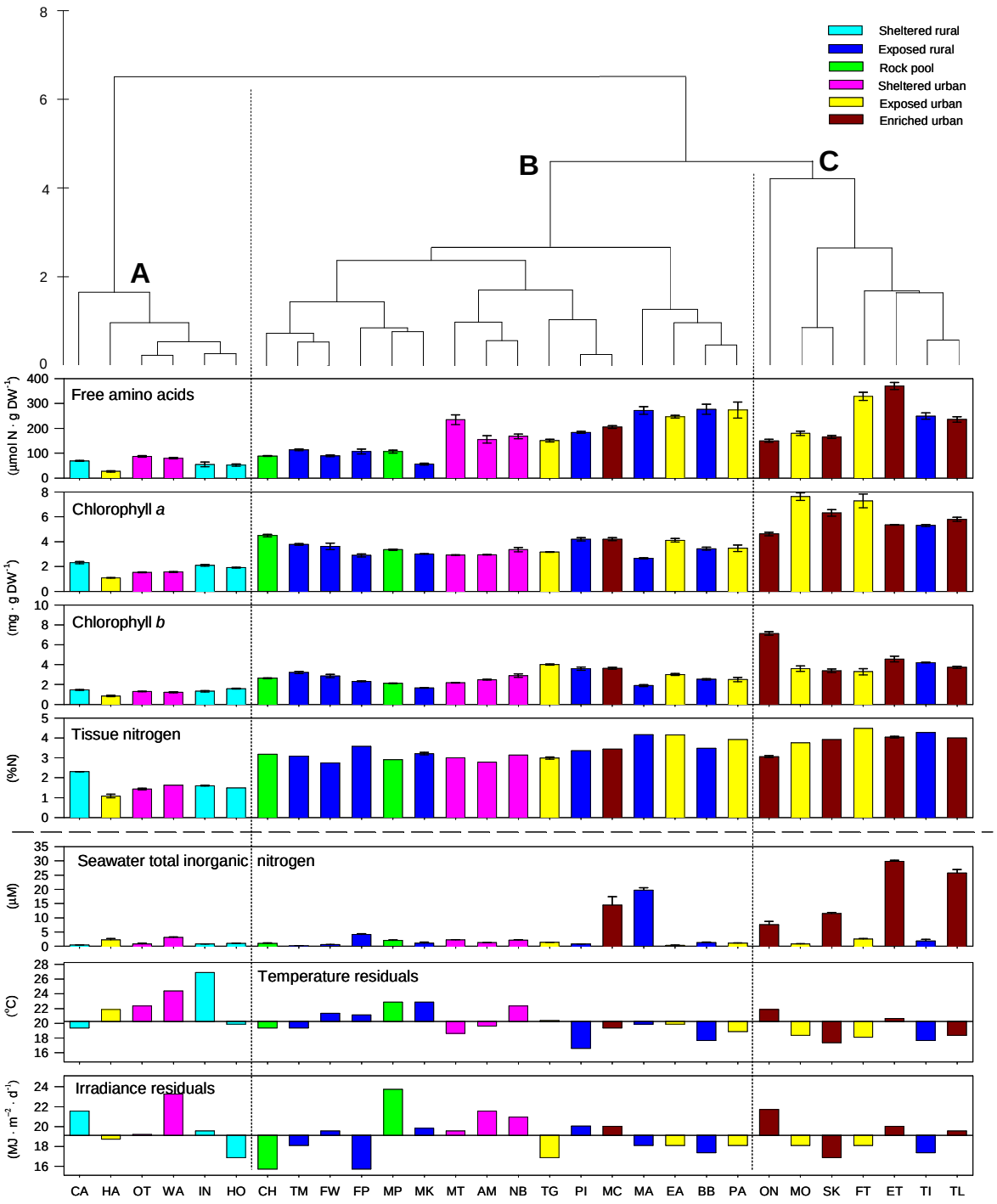



**Figure 2.8.** Similarity dendrogram plot of (normalised) Euclidian distances between five N-indices measured in 29 *Ulva* populations in winter 2002. Three main groupings (A, B and C) are separated by vertical dashed lines. Clustered groups of sites range from those with (A) *Ulva* containing low N-indices, through an intermediate group of sites with (B) *Ulva* containing intermediate N-indices, to a group of sites with (C) *Ulva* containing high N-indices. Histograms of the five *Ulva* N-indices used to construct the dendrogram are shown below the cluster plot. Values for seawater total inorganic nitrogen concentration, and residual values calculated for seawater temperature and surface irradiance are shown in the bottom two histograms.

with a very different biochemical expression of nitrogen metabolism, although this seems unlikely.

Although variable, median seawater TIN concentrations were significantly different ($P < 0.05$) between the three groups (based on Kruskal-Wallis one-way analysis of variance on ranks). Median seawater TIN concentrations ranked in order: A < B < C at 1.2 μM, 2.0 μM and 10.3 μM, respectively. There was no significant difference in either monthly averaged surface irradiance ($P = 0.44$) or in seawater temperature ($P = 0.83$) across the three groups (Figure 2.7, bottom two histograms).

***Winter***

As with the summer comparison, similarity of winter *Ulva* N-indices resulted in three main groups. Going from left to right on Figure 2.8 these groups ranged from sites with *Ulva* containing low (Figure 2.8, A), medium (Figure 2.8, B), and high N-indices (Figure 2.8, C). Group A included three sheltered rural sites (CA, IN and HO), one exposed urban site (HA), and two sheltered urban sites (WA and OT). Group B contained close to half of all sites in the winter survey and included both rock-pool sites, most of the open coastal sites and most of the urban (exposed and sheltered) sites including one enriched urban site (MC). Group C (Figure 2.8, C) contained the four remaining enriched urban sites, two exposed urban sites close to Wellington (MO and FT) and one exposed rural site (TI) (Figure 2.8, C). As with the summer survey *Ulva* from Onehunga lagoon formed a sub-group within this high tissue-N group. Another notable feature of the data was that besides *Ulva* from enriched urban environments, *Ulva* that came from exposed sites (urban and rural alike as shown by blue and yellow symbols in Figure 2.8) also tended to have high tissue-N indices. As with summer it is worth noting that asparagine was a dominant amino acid in *Ulva* that also had high N-indices and was present in high levels in most samples from group B and all from group C (Appendix 2.10).

Median seawater TIN concentrations ranked in order: A < B < C at 0.9 μM, 1.3 μM and 7.5 μM, respectively, however according to a Kruskal-Wallis one-way analysis of variance on ranks there was probably no significant difference ($P = 0.06$) between the three groups. In addition there was also no significant difference ($P = 0.60$) in light across the three groups, but temperature was significantly lower ($P < 0.05$) at 11.5 ˚C

in group C compared with either groups A or B (which had mean seawater temperatures of 15.3 ˚C and 12.9 ˚C, respectively).

### 2.4.5 Season, environment and *Ulva* N-status

Across all sites there was no overall significant difference ($P$ = 0.26) in seawater TIN concentrations between seasons (Appendix 2.11). Conversely, *Ulva* tissue-N indices (including proline (Pro), Asn, FAA, TChl and TN) were significantly higher (around 2-fold) in winter than in summer (Appendix 2.11). On the other hand, there was no overall significant difference in chlorophyll *a* : *b* values or in $\delta^{15}N$ values between summer and winter (Appendix 2.11). The most pronounced seasonal shift in any biochemical response measured in *Ulva* was Pro which was on average five-fold higher in winter (22.8 µmol N · g $DW^{-1}$) than summer (4.7 µmol N · g $DW^{-1}$) (Appendix 2.11). There was also a broad negative relationship between seawater temperature and proline suggesting decreasing temperature was linked to accumulation of proline (Figure 2.9).

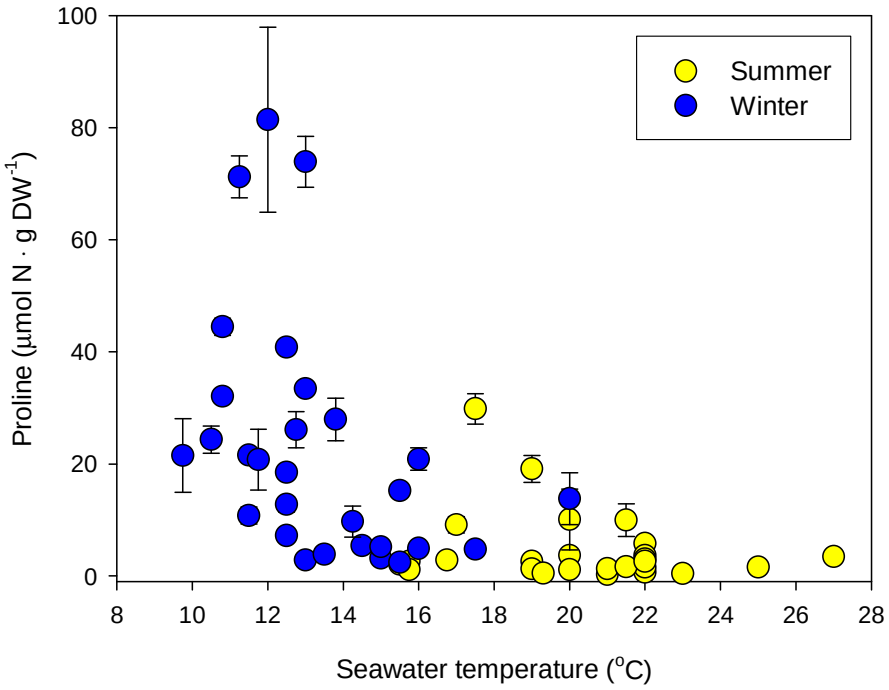



**Figure 2.9.** Change in *Ulva* proline levels with change in seawater temperature. Values for proline are means ± S.E. for three replicate samples.

Given that seasonal factors other than seawater TIN concentration were clearly implicated in the nitrogen status of *Ulva*, the relationships between *Ulva* N-indices, and seawater temperature and surface irradiance, were examined. Using data pooled from both seasons there were weak, negative correlations between both seawater temperature and surface irradiance, and TN, TChl and FAA content (data not shown). However, out of the three indices TN showed the strongest overall correlation with temperature ($R^2$ = 0.41) and irradiance ($R^2$ = 0.34) (data not shown). TN levels in *Ulva* from the three main ordination groups (A, B and C) in Figure 2.7 and 2.8 were compared with seawater temperature for combined summer and winter data. In addition, *Ulva* at exposed sites, particularly those in more southern waters in the winter, contained more nitrogen (sometimes grouping with other nitrogen-enriched *Ulva* (see Figure 2.8)). Therefore exposed sites were treated separately from *Ulva* in sheltered sites in the plots below.

TN content in *Ulva* from the three ordination groups all showed negative relationships with seawater temperature, however the *y*-intercept values were different for the three groups (Figure 2.10, A). The average slope for sheltered sites showed there was an average 668 μmol · g $DW^{-1}$ (calculated from 0.94 % N) change in TN content in *Ulva* with a 10 ˚C decrease in water seawater temperature (Table 2.8). *Ulva* growing on exposed sites on average showed a more dramatic 1356 μmol · g $DW^{-1}$ (calculated from 1.9 % N) increase in TN content with a 10 ˚C decrease in water seawater temperature (Figure 2.10, B and Table 2.8).

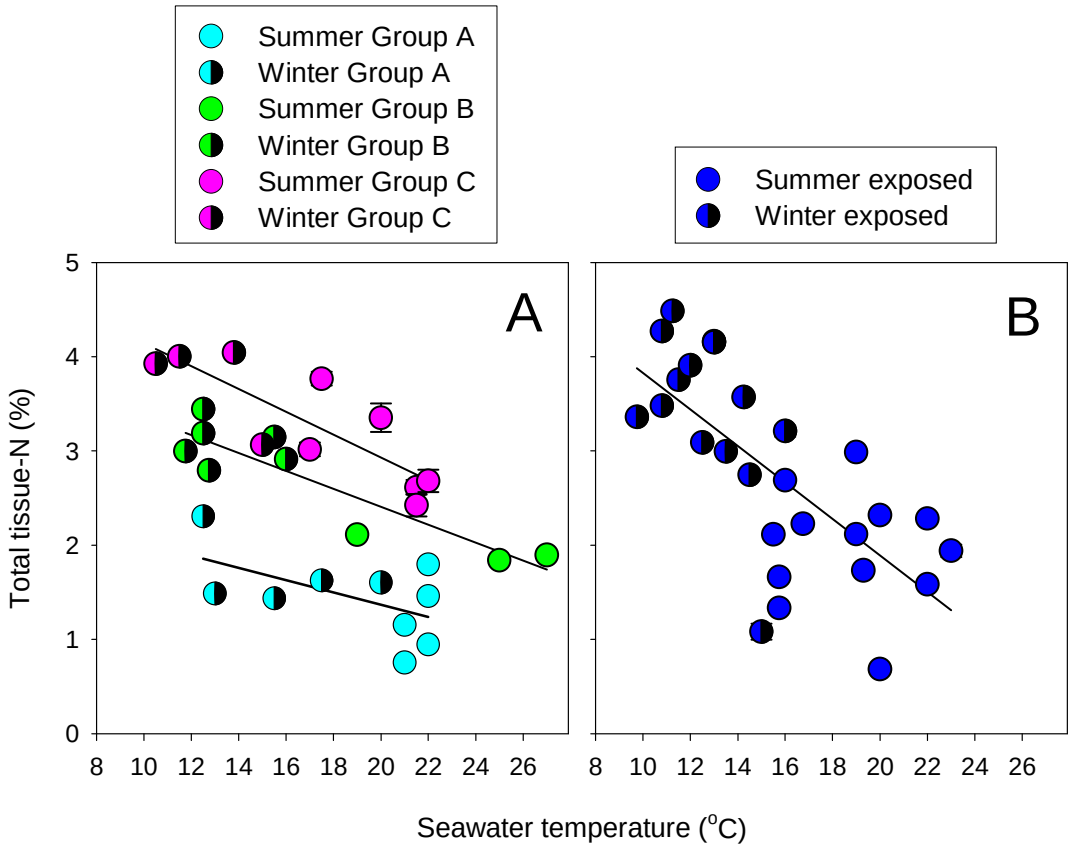



**Figure 2.10.** (**A**) Change in *Ulva* total tissue nitrogen content with change in seawater temperature from three groups of sheltered sites (based on three dendrogram groupings in Figure 2.7 and 2.8, A, B and C). (**B**) Change in *Ulva* total tissue nitrogen content with change in temperature from exposed sites only. Linear regression lines are fitted using ordinary least squares.

**Table 2.8.** Regression statistics for relationships between seawater temperature and total tissue nitrogen content in *Ulva* from three ordination-based groups (A, B and C) in Figure 2.7 and 2.8 and *Ulva* from exposed sites.

| | $R^2$ | *P-value* | *df* | *F* | slope | *Y-intercept* |
|---|---|---|---|---|---|---|
| **Group A** | 0.31 | 0.096 | 1,9 | 3.56 | -0.065 | 2.66 |
| **Group B** | 0.81 | <0.001 | 1,8 | 30.54 | -0.095 | 4.31 |
| **Group C** | 0.70 | 0.003 | 1,9 | 18.23 | -0.121 | 5.35 |
| Average slope for group A, B and C | | | | | **-0.094** | |
| **Exposed** | 0.495 | <0.0001 | 1,26 | 24.466 | **-0.194** | 5.768 |

Qualitative environmental factors were further examined using three-way analysis of variance. Turbulence and bulk water motion should be higher on exposed or semi-exposed coasts relative to sheltered bays and estuaries. Therefore, comparisons were made between seasons of TN levels in *Ulva* from exposed and sheltered sites, and urban and rural settings as factors. Data from enriched urban sites were excluded from analysis. In addition, since Wellington Harbour was over-represented, two sites (FT and PA) were randomly withdrawn from analysis.

There was a significant effect of both season ($P < 0.001$) and exposure ($P = 0.001$) on TN content in *Ulva* around New Zealand (Table 2.9, A). TN content in *Ulva* was highest at exposed sites, particularly in the winter (Figure 2.11, A), but there was no significant effect of season, exposure, or urban/rural setting on seawater nitrogen concentrations which might explain this (Table 2.9, B). Levels of TN content in *Ulva*

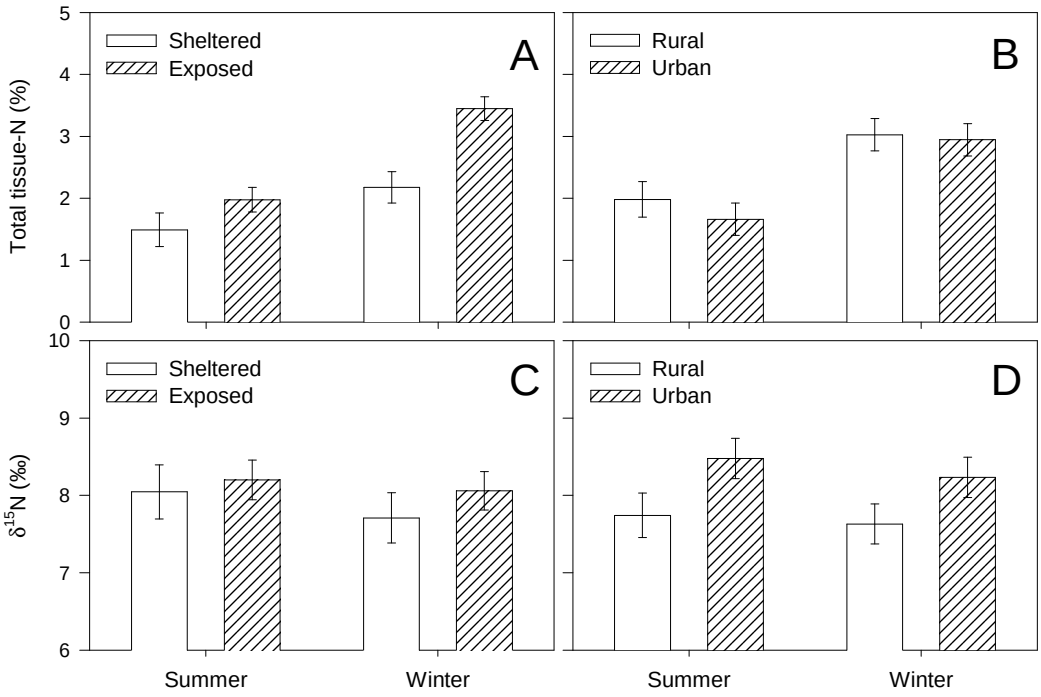



**Figure 2.11.** Comparison of least squares means (derived from Holm-Sidak pair-wise comparisons) for (**A** and **B**) total tissue nitrogen content, and **(C** and **D)** $\delta^{15}N$ values in *Ulva* from sheltered and exposed sites (**A** and **C**), and rural and urban sites (**B** and **D**) in summer and winter.

**Table 2.9.** Three-way general linear model analysis of variance for (**A**) total tissue nitrogen content in *Ulva*, (**B**) seawater inorganic nitrogen concentration and (**C**) $\delta^{15}N$ content in *Ulva* versus environmental factors and season (summer and winter).

**A) Three-way ANOVA on differences in *Ulva* nitrogen (TN) content versus urban or rural location, exposure and season**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Season | 1 | 9.28 | 9.28 | 20.46 | <0.001 |
| Exposure | 1 | 5.90 | 5.90 | 13.02 | 0.001 |
| Urban/Rural | 1 | 0.00 | 0.00 | 0.01 | 0.938 |
| Season x Exposure | 1 | 0.71 | 0.71 | 1.56 | 0.221 |
| Season x Urban/Rural | 1 | 0.01 | 0.01 | 0.02 | 0.876 |
| Exposure x Urban/Rural | 1 | 3.54 | 3.54 | 7.80 | 0.009 |
| Season x Exposure x Urban/Rura | 1 | 0.10 | 0.10 | 0.22 | 0.639 |
| Residual | 31 | 14.05 | 0.45 | | |
| Total | 38 | 36.67 | 0.97 | | |

**B) Three-way ANOVA on differences in seawater TIN concentration versus urban or rural location, exposure and season**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Season | 1 | 10.84 | 10.84 | 0.67 | 0.419 |
| Exposure | 1 | 0.93 | 0.93 | 0.06 | 0.812 |
| Urban/Rural | 1 | 1.49 | 1.49 | 0.09 | 0.764 |
| Season x Exposure | 1 | 5.04 | 5.04 | 0.31 | 0.581 |
| Season x Urban/Rural | 1 | 0.47 | 0.47 | 0.03 | 0.866 |
| Exposure x Urban/Rural | 1 | 35.34 | 35.34 | 2.19 | 0.149 |
| Season x Exposure x Urban/Rura | 1 | 0.39 | 0.39 | 0.02 | 0.877 |
| Residual | 31 | 501.15 | 16.17 | | |
| Total | 38 | 565.56 | 14.88 | | |

**C) Three-way ANOVA on differences in *Ulva* $\delta^{15}N$ content versus urban or rural location, exposure and season**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Season | 1 | 0.17 | 0.17 | 0.22 | 0.639 |
| Exposure | 1 | 1.35 | 1.35 | 1.81 | 0.187 |
| Urban/Rural | 1 | 4.04 | 4.04 | 5.42 | 0.026 |
| Season x Exposure | 1 | 0.01 | 0.01 | 0.02 | 0.894 |
| Season x Urban/Rural | 1 | 0.15 | 0.15 | 0.20 | 0.660 |
| Exposure x Urban/Rural | 1 | 0.56 | 0.56 | 0.75 | 0.394 |
| Season x Exposure x Urban/Rura | 1 | 0.44 | 0.44 | 0.59 | 0.448 |
| Residual | 34 | 25.35 | 0.75 | | |
| Total | 41 | 33.34 | 0.81 | | |

from urban and rural sites were not significantly different, allowing for the effects of season ($P$ = 0.876) (Table 2.9, A and Figure 2.11, B). However, there was a significant interaction of the effects of exposure and urban/rural setting on levels of

TN in *Ulva* ($P$ = 0.009) (Table 2.9, C) (see also Appendix 2.12). This suggests that the level of TN content in *Ulva* from exposed and rural environments might have been to some degree dependent on whether sites were located in urban or rural environments. In contrast to the differences in TN content in *Ulva* between exposed and sheltered sites, there was no significant effect of either season ($P$ = 0.639) or exposure ($P$ = 0.187) on $\delta^{15}$N values (Table 2.8, C and Figure 2.11, C). The only significant effect ($P$ = 0.026) of rural or urban setting on any measured N-indices was in slightly higher $\delta^{15}$N values in *Ulva* from urban sites (Table 2.8, C and Figure 2.11, D).

### 2.4.6 *Ulva* taxonomy and environment in relation to nitrogen status.

Comparisons of two morphologically distinct groups of *Ulva* in relation to nitrogen loading and environment were made (Figure 2.12). *U. intestinalis* (formerly *Enteromorpha intestinalis* (Blomster *et al.*, 1998)) with monostromatic, tubular thalli (cross-hatched bars), and *U. fasciata* (Maggs and McIvor, 2004) and another unidentified species of *Ulva* (possibly *U. fasciata)* with distromatic, frondose thalli (solid bars) were compared between two sites with contrasting nutrient loading. Note that at both sites the two distinctive morphologies occurred growing side-by-side attached to the substrate. These sites consisted of a low N-loading site at Inner Beach (IN, sheltered rural) and a high N-loading site at Ebbtide Road (ET, enriched urban) (Figure 2.12, bottom plot).

The most obvious differences in *Ulva* N-indices between the two contrasting sites were in levels of total free amino acids, asparagine, tissue-N content, chlorophyll *b* (and to a lesser extent chlorophyll *a*) and $\delta^{15}$N. In some cases values of N-indices appeared to be related to environmental differences and irrespective of differences in species and morphology (Figure 2.12). However, there were some important differences. While *U. intestinalis* from both sites and *Ulva sp.* from the IN site had similar chlorophyll *a* : *b* ratios, *U. fasciata* had a much lower ratio. The ratio of chlorophyll *a* : total tissue-N was not site-specific, but apparently related to either algal morphology or possibly genetic factors given the close similarity between the two *U. intestinalis* samples (Figure 2.12).

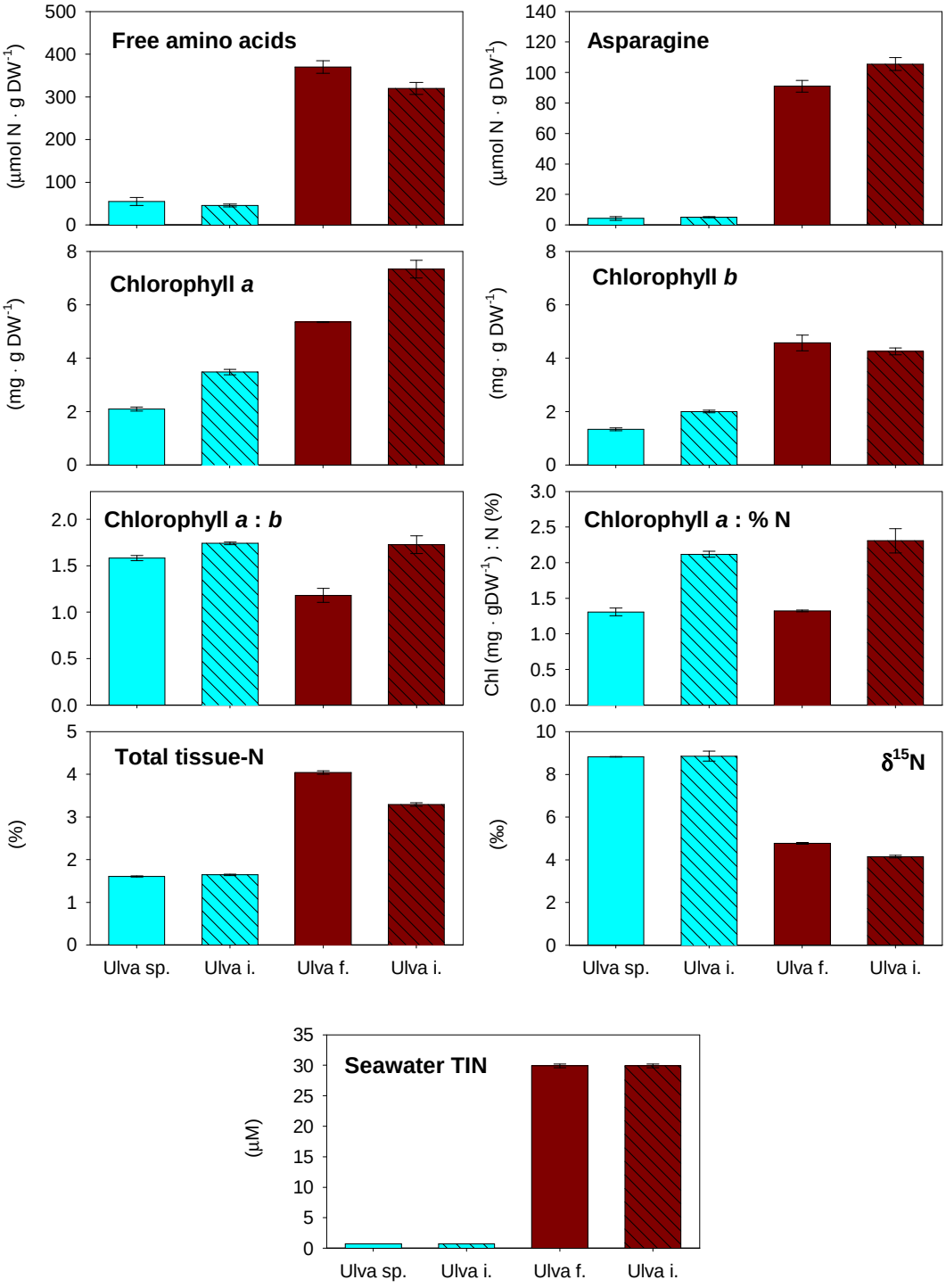



**Figure 2.12.** Comparison of N-indices in morphologically distinct *Ulva* species growing side-by-side at two contrasting sites: Inner Beach, Farewell Spit (blue bars) and Ebbtide Road, Christchurch (brown bars). *Ulva intestinalis* = Ulva i. (cross-hatched fill), *Ulva fasciata* = Ulva f. (solid fill) and *Ulva species* = Ulva sp. (solid fill). Values are means ± S.E. for three replicate samples.

Similarity of *Ulva* nitrogen status (based on total free amino acids, total chlorophyll and tissue-N content) in relation to both environmental and taxonomic grouping was also examined. Both summer and winter data were examined, however since there

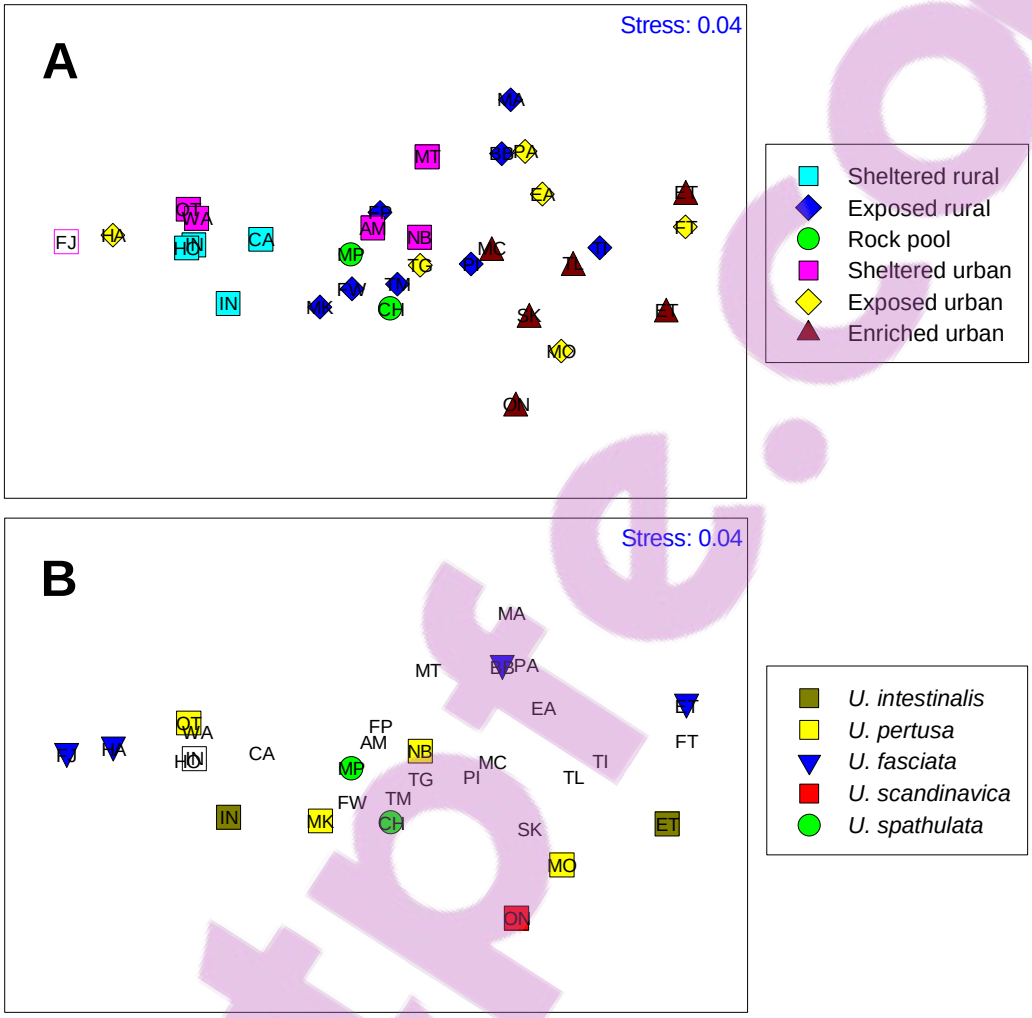



**Figure 2.13.** Similarity of nitrogen status in *Ulva* samples (based on MDS plots of total free amino acid, total chlorophyll and tissue nitrogen content) from contrasting environments around New Zealand in winter 2003. Note that a sample of *Ulva fasciata,* which was collected from Tokoriki Island, Fiji (FJ, located at the extreme left-hand side of the plots) in late winter 2004, has been included. Symbols in the top plot indicate environmental categories while symbols in the bottom plot indicate *Ulva* species (where known) based on either distinctive morphology or close homology of the *rcb*L gene with sequences held in GenBank. Note also that the unfilled square in the bottom plot is the unidentified species used in the comparison between *Ulva* samples collected from the Inner Beach (IN) and Ebbtide road (ET) sites, which contrasted in levels of nitrogen loading.

were more species collected and identified from the winter survey (including one sample of *U. fasciata* collected from Fiji in the late winter of 2004 as well as the samples of *U. intestinalis* examined above in Figure 2.12), only this data was presented.

Multi-dimensional scaling (MDS) plots showed that there was a general tendency for most members of environmental categories to group together, particularly those from sheltered rural sites, rock pools and enriched urban sites (Figure 2.13, A). On the other hand most taxonomic groups with the exception of *U. spathulata* showed a broader distribution in two-dimensional space (Figure 2.13, B) (note that there was only one representative of *U. scandinavica* from Onehunga lagoon). For example, while *U. pertusa* showed a reasonable distribution across the MDS plots, *U. fasciata* showed the widest distribution with examples of very low N-status algae from Napier (HA) on the left of the plots through to a high N-status example from the Ebbtide Road (ET, enriched urban) in Christchurch on the right (Figure 2.13, B). In addition, both *U. pertusa* and *U. fasciata* showed a similarly broad distribution in relation to N-status in the summer (Appendix 2.13). Finally, the grouping of the morphologically distinct *Ulva* examples from Inner Beach (IN) and Ebbtide Road (ET) (examined above in Figure 2.12) in the winter suggested that the alga's relative position in two-dimensional space (Figure 2.13, B) was largely due to environmental rather than taxonomic difference.

## 2.5 Discussion

***Seawater nutrients***

Excluding enriched urban sites, in most coastal situations around New Zealand seawater nutrients were variable and low in the order of 2 - 3 µM, and in many cases below 1 µM (e.g., sheltered rural environments). In contrast, there were two clear examples on exposed rural coasts (Paia Point (PI), in Kaikoura, in the summer and Makara (MA), near Wellington, in the winter) where seawater TIN concentrations were high (14 to 19 µM). Being mostly comprised of nitrate ($NO_3^-$), these elevated levels were probably attributable to upwelling, certainly in case of the Paia Point site given it was sampled during a heavy storm (7 February 2002).

Phosphate ($PO_4^{3-}$) concentrations in seawater were typically low in both summer and winter at around 0.5 µM or less in all categories except the enriched urban sites. This suggests that $PO_4^{3-}$ might be a useful seawater parameter for indicating an urban contribution to seawater nutrients in impacted sites. However, the N : P ratio was typically variable, but often well under 30 : 1 suggesting that $PO_4^{3-}$ was probably not in limiting supply for algal growth in most coastal situations (Atkinson and Smith, 1983). The only exception to this was in the two rock pools sampled in the summer with N : P of 42.6 ± 7.4. This may have represented the only situation in this survey where P was present in limiting concentrations.

In many cases in this survey ammonium ($NH_4^+$) was the dominant form of inorganic nitrogen in coastal environments, but this was particularly noticeable in enriched urban sites and rock-pools in the summer. Sharp (1983) suggests that $NH_4^+$ concentrations in coastal areas are usually below 3 µM, but terrestrial sources, such as sewage, may increase values to 25 µM or more. High $NH_4^+$ concentrations measured in many of the enriched urban sites, particularly at the Avon-Heathcote estuary in the summer, were almost certainly derived from sewage or wastewater effluent (Bolten-Ritchie and Main, 2005). According to Sharp (1983) $NH_4^+$ concentrations in coastal zones typically comprise less than 20 % of total inorganic N in seawater. However, on average $NH_4^+$ represented a higher percentage of TIN in New Zealand coastal waters, both in summer (64.0 ± 5.4 %) and winter (51.6 ± 5.5 %). Nutrient data from the University of Auckland Leigh Marine Laboratory from 2000 to 2004 would tend to support these values with average $NH_4^+$ / TIN values of 44.9 ± 1.3 % (Dobson, unpublished data). This higher contribution of $NH_4^+$ to TIN may have a consequence for seaweeds around New Zealand since it is generally thought to be the preferred inorganic nitrogen source in seaweeds (D'Elia and DeBoer, 1978; Raven *et al.*, 1993; Rees, 2003; Cohen and Fong, 2004a) with many, often opportunistic, seaweeds showing a higher uptake capacity for this nutrient compared with nitrate (Thomas and Harrison, 1987; Rees, 2003; Cohen and Fong, 2004a).

***Environment, season and N-content in* Ulva**

*Seawater nitrogen concentration. Ulva* collected from enriched urban sites in summer had the highest mean levels of all measured N-indices (particularly FAA and Asn).

Conversely, *Ulva* collected from sheltered rural sites with low seawater TIN concentrations, in nearly all cases had the lowest N-indices. These observations alone suggested that the N-indices in *Ulva* examined in this study integrate high levels of seawater nitrogen loading, despite the probable variability in measured seawater nutrients (see Appendix 2.14 for an example of long term seawater nitrogen variability in the Avon-Heathcote Estuary). Moreover, the comparison made between two distinct groups of *Ulva* from the two environments that contrasted in levels of nitrogen loading (Figure 2.13), suggested that it was mainly the environment, as opposed to taxonomic difference, that dictated the levels of N-indices observed in *Ulva*. It would also appear that taxonomy was less important in determining relative nitrogen status of *Ulva* compared with the effect of environment. *Ulva fasciata* was found in very different environments in this survey and also showed extremes of nitrogen status. Similarly *U. intestinalis* and *U. pertusa* also showed a range of nitrogen status in different environments. On the other hand the two examples of *U. spathulata* from geographically well separated sites grouped closely in both summer and winter. It is possible that this simply reflects the fact that they were collected from a similar relative intertidal position. It is proposed that nitrogen metabolism, as reflected by most tissue N-indices in *Ulva*, appears to be fundamental and conserved within this genus (see Chapter Five for further discussion).

In this study there were examples of sheltered urban sites, for example Whangamata (WA), which had similar levels of nitrogen indices to *Ulva* growing in the sheltered rural sites in the South Island. Another sheltered urban site that had *Ulva* with lower N-indices (relative to *Ulva* in enriched urban sites) was Otumoetai (OT) in Tauranga Harbour. In the winter *Ulva* at this site had low N-indices (Figure 2.8) which presumably reflected average lower levels of nitrogen availability. At this time the site had both concentrations of seawater inorganic nitrogen and N-indices in *Ulva* that contrasted with measurements made at the Avon-Heathcote estuary (ET) (Appendix 2.4 to 2.7). Both of these harbours have historically suffered from *Ulva* blooms and the question arises: is this because both areas suffer from high levels of nutrient enrichment? The answer appears to be no and it is quite probable that other factors (including other nutrients such as phosphorus) are involved in these *Ulva* blooms. However, the possibility that only minor increases in nitrogen supply might be a very important factor in *Ulva* blooms in Tauranga Harbour can not be discounted.

Moderately high $NH_4^+$ concentrations (3 - 11 µM) were recorded in the summer in the two rock-pools (CH and MP). These were both well-populated with *Xenostrobus* mussels in both seasons, and it is likely that a significant proportion of the $NH_4^+$ measured in these seawater samples was derived from excretion by these animals. According to statistical ordination (Figure 2.7 and 2.8) *U. spathulata* from both rock pools were moderately enriched in both summer and winter. These environments represented a very different situation relative to most other *Ulva* populations in this survey. The pools were located in the high intertidal and were therefore only supplied with fresh seawater at high tide or when weather conditions were rough. Their low phosphorus concentrations might also have indicated that phosphate supply was primarily controlled by seawater replacement. In terms of nitrogen it has been shown that nitrogen derived from closely associated fauna can be important for macroalgae. The benefit to macroalgae of nitrogen derived from closely associated fauna has been shown in studies of sessile organisms, including barnacles (Williamson and Rees, 1994) and bryozoans (Hurd *et al.*, 1994a) and suggested in the case of mobile epifauna (Taylor and Rees, 1998). Moreover, *Ulva* grown in the presence of mussels had 30 – 100 % greater nitrogen content compared with *Ulva* grown in isolation (unpublished data).

*Effects of exposure, water motion and season.* Other than the high levels of N-indices in *Ulva* from nitrogen-enriched sites in the summer, one of the clearest differences between categories was in *Ulva* sampled from exposed and sheltered sites (Figure 2.11, A). In contrast to exposed or semi-exposed coastal sites, *Ulva* from low-nutrient, sheltered sites had a lower nitrogen status in both seasons. *Ulva* growing in these environments was more likely to become nutrient limited (Parker, 1981; Lin and Hung, 2004) due to diffusive boundary layer formation (Wheeler, 1988; Hurd, 2000). Conversely in the enriched urban sites, which (except for the Saint Kilda (SK) discharge site) were all classified as sheltered, it might be expected that high nutrient concentrations would tend to compensate for low water motion, as shown by Parker (1981). Various studies have clearly demonstrated links between nitrogen loading in sheltered environments and changes in macroalgal communities (including those resulting in *Ulva* blooms) and have been well reviewed by Cloern (2001).

Compared with *Ulva* from sheltered sites, *Ulva* growing in exposed coastal sites should be subject to higher levels of turbulence and bulk water flow. This in turn should result in higher rates of uptake of nitrogen (and other nutrients) in macroalgae, as suggested by several authors (e.g., Parker, 1981; Hurd *et al*., 1996; Larned and Atkinson, 1997; Hurd, 2000; Smit, 2002), and therefore higher levels of N-indices. There was a stronger effect of exposure on TN content in the winter which could simply be interpreted as more water motion and turbulence because of rougher sea conditions in the winter. However, because of the haphazard nature of the survey it is possible that southern sites were over-represented (because *Ulva* was more abundant in southern New Zealand). Moreover, urban sites, both exposed and sheltered alike, tended to have a more northern distribution. In addition, there was also a significant interaction of the effects of exposure and urban/rural setting on levels of TN content in *Ulva* suggesting that to some degree the effect of exposure was dependent on whether sites were located in urban or rural environments. Therefore it is possible that these observations were partly attributable to design imbalance.

In the winter *Ulva* from the exposed rural and urban sites, which grouped with *Ulva* from the enriched urban sites (see Figure 2.8), all came from either the east coast of the South Island or from around Wellington. These sites, as well as potentially having a higher supply of nutrients from either upwelling (i.e., east coast of the South Island) or sources associated with large urban environments (i.e., Wellington Harbour), were also colder on average than most other sites in the country. Because of the relationship with temperature and light (Figure 2.10, A and B) it is suggested that colder, lower light conditions in winter coupled with the effects of water motion on exposed shores in winter had an additive effect on tissue-N content in *Ulva*. Under conditions of low energy and low growth potential (e.g. winter) nitrogen uptake and assimilation should lead to a surplus in internal stores of nitrogen that is in excess of the requirements for growth. This may also to a large degree explain the overall higher levels of nitrogen content in *Ulva* observed in the winter.

*Effects of nitrogen loading and season.* Light and temperature are fundamental to growth in seaweeds. Factors affecting growth in *Ulva* are well documented for light (Duke *et al.*, 1989a; Coutinho and Zingmark, 1993; de Casabianca *et al.*, 2002) and temperature (Steffensen, 1976; McLachlan and Bird, 1986; Duke *et al.*, 1987; Duke

*et al.*, 1989a; Fredriksen and Rueness, 1989; Laing *et al.*, 1989; de Casabianca and Posada, 1998). The importance of temperature on growth in *Ulva* was demonstrated by Henley and Ramus (1989b) who stated that "temperature changes of 2 or 3 ˚C had a much greater effect" on growth than did the constant addition of 8 - 12 µM $NH_4^+$ (Henley and Ramus, 1989b). Moreover, several studies have highlighted the importance of interactions between light, temperature and nitrogen supply (Duke *et al.*, 1987; Duke *et al.*, 1989b; Duke *et al.*, 1989a; Rivers and Peckol, 1995; de Casabianca and Posada, 1998; Altamirano *et al.*, 2000; Taylor *et al.*, 2001). As suggested by Duke et al (1989b) "the proximal mechanism for seaweeds' accumulation of N at low light and temperatures may be that N uptake is less limited by light and temperature than is growth".

The effects of light and temperature couldn't be separated in the current study (because they naturally co-vary), and the light measurements used were crude and probably did not reflect light that occurred at the thallus surface after passing through the water column. However, if the data set from this survey is revisited using the original subjective categories for sheltered sites, the relationship between *Ulva* TN and temperature is very similar to that in Figure 2.10 and TN in *Ulva* from three categories also independently correlated with both temperature and light (Figure 2.14 below). This not only might suggest that the original selection criteria were reasonable, but also that if *Ulva* growing in sheltered environments is found to have high TN (or possibly FAA) content this may indicate that the alga is integrating on average higher levels of N-loading despite, apparently, seasonal differences in temperature and light. It is suggested that the three categories of *Ulva* shown below represent environments that are stable in terms of biologically available nitrogen. The category that showed the greatest variability in this respect was that of the urban sites (shown as text labels). It is highly probable that this variability reflects different levels of biologically available nitrogen in these urban environments. However, it is also acknowledged that there are many other factors that weren't examined in this study that may also alter how *Ulva* reflects nitrogen availability. These include site-specific information on actual average nitrogen loading, light (and water column turbidity), temperature and water motion. In addition, the amount of biomass present may also have a direct influence on concentrations of nitrogen in the seawater column

(as suggested by Thybo-Christesen *et al*., 1993 and Flindt *et al*., 1999), and therefore on TN content in *Ulva* thalli growing in dense algal stands.

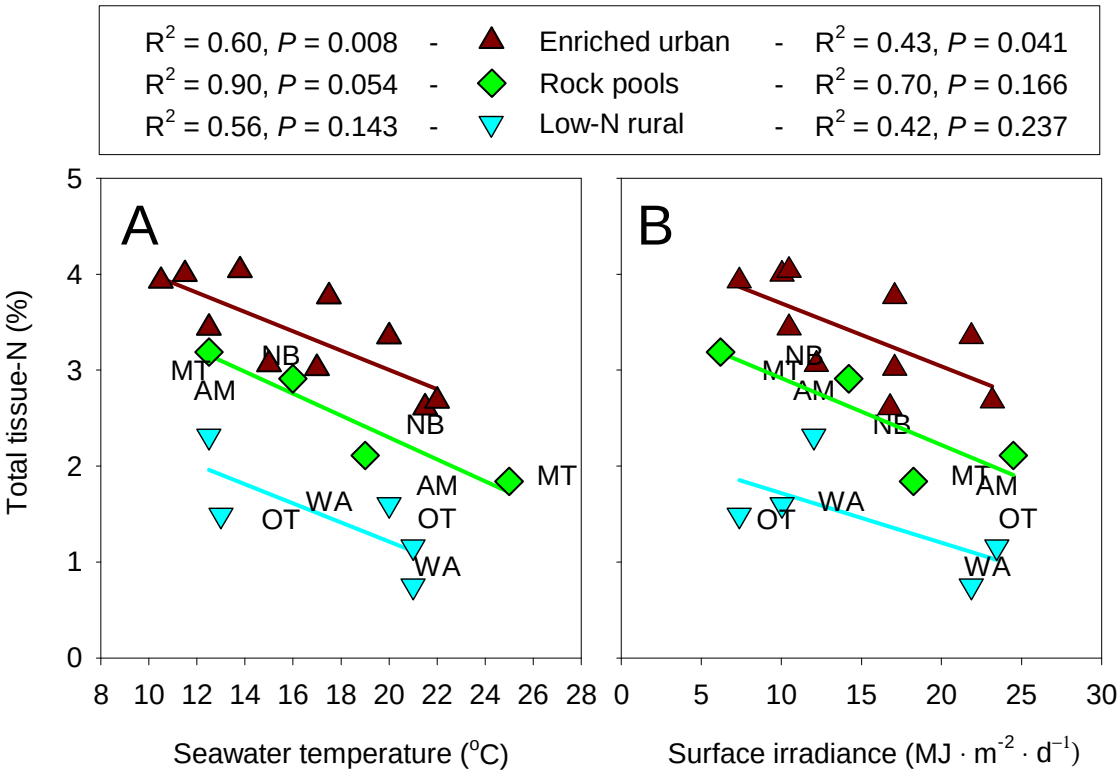



**Figure 2.14.** Change in *Ulva* total tissue nitrogen content in *Ulva* from enriched urban, rock pools, sheltered rural and urban sites with change in (**A**) seawater temperature and (**B**) surface irradiance. Linear regression lines are separately fitted to *Ulva* from enriched urban sites, rock pools and sheltered rural sites. Regression statistics are located in the legend above each plot. Sheltered urban sites are included as text symbols only.

### *Free amino acids in* Ulva

In this study FAA pools in a selection of natural *Ulva* populations represented 2.1 to 12.8 % of TN (Appendix 2.5 and 2.7). This range was slightly greater than values (4 and 9 %) in a study of *Ulva fenestrata* by Naldi and Wheeler (1999). The dominant amino acid at enriched urban sites, and many exposed sites in the winter, was asparagine with the highest value (182 µmol N · g $DW^{-1}$) in the survey recorded in *Ulva* from the Avon-Heathcote estuary. On the other hand glutamine, being a key amino acid involved in nitrogen metabolism, although generally higher at enriched

urban sites was typically found in low levels (< 5 μmol N · g $DW^{-1}$). These values were in contrast to values (70 μmol N · g $DW^{-1}$) of glutamine recorded in nitrogen enriched (22 μM) intertidal *Ulva intestinalis* in the study of Barr & Rees (2003). However, the highest seawater TIN concentration (110 μM) recorded in this survey (at Ebbtide road, ET) only resulted in a glutamine value of 2.4 μmol N · g $DW^{1}$ in *Ulva*. It is possible that in these *Ulva* populations, either glutamine is quickly converted to glutamate (which in turn is quickly utilised for synthesis of other amino acids) or that these low levels may result from down-regulation of nitrogen assimilation given these essentially subtidal algae have constant access to external nitrogen.

While these results suggest that asparagine was an important storage amino acid, it should also be noted that not all *Ulva* produce asparagine in any significant amount when enriched with ammonium. For example, there was no significant increase in asparagine levels in either *U. spathulata* from rock pools or *U. pertusa* from the Mokohinau Islands (MK) when enriched with 10 μM ammonium in outdoor cultures (unpublished data). In contrast, *U. pertusa* from Otumoetai (OT) maintained under identical conditions at the same time as *U. pertusa* from the Mokohinau Islands produced increased levels of asparagine, suggesting that the control of asparagine synthesis was more complex than *Ulva* taxonomy would suggest (unpublished data).

The amino acid proline showed the greatest (five-fold) increase in winter, relative to summer, of any biochemical parameter examined in this survey. Although it wasn't a major contributor to the FAA pool (about 14 % in winter) this change suggests that it had an important metabolic role in *Ulva* in the winter. Proline accumulation is a common metabolic response to water deficits, and salinity stress or temperature stress in higher plants (Matysik *et al.*, 2002) and algae (Kakinuma *et al.*, 2006). Proline accumulation in algae has also been shown to be a stress response to high levels of heavy metal contamination (Sharma and Dietz, 2006). In the study of Kakinuma *et al* (2006) proline accumulation in *Ulva* was shown to occur with high temperature stress. In higher plants, however, increases in proline are also associated with cold acclimation in winter (Dörffling *et al.*, 1997). It seems likely that the changes in proline levels in *Ulva* in the winter represent some form of cold acclimation response. Whatever the ultimate cause of increased proline in the winter, it is clearly

fundamental to the biology of *Ulva*, and additionally, links the effects of abiotic factors (most probably temperature) to nitrogen content in *Ulva*.

***Survey of tissue $\delta^{15}N$ isotopes in* Ulva**

Some studies have shown that $\delta^{15}N$ values in macroalgal tissues greater than about 9 ‰ (McClelland *et al.*, 1997; Jones *et al.*, 2001; Gartner *et al.*, 2002; Dudley, 2005) may indicate the presence of effluent, including secondary or tertiary treated sewage as a source of nitrogen which has undergone some denitrification (Rogers, 2006). Other studies have shown that untreated wastewater sources can result in ratios approaching 2 ‰ (Rogers, 1999; Dudley, 2005) potentially due to a dominance of industrially-derived nitrates with more negative values (Rogers, 2006). *Ulva* from enriched urban sites in the current study showed the largest range of $\delta^{15}N$ values in both seasons, suggesting that these extreme values were the result of differences in nitrogen isotopic composition in the environment. However, $\delta^{15}N$ values alone may not be reliable indicators of high nitrogen loading *per se*, but were, in the context of this survey, potentially useful as qualitative indicators of differences in nitrogen composition and source. In contrast to enriched urban sites, there was a narrow range of $\delta^{15}N$ values in *Ulva* from all rural sites regardless of geographic location or season. It was possible that the higher $\delta^{15}N$ values recorded in urban sites in both seasons indicated the contribution of nitrogen enriched in $^{15}N$ from either secondary or tertiary treated sewage effluent. Alternatively, higher $\delta^{15}N$ values seen in some urban environments may also have indicated fractionation associated with denitrification (theoretically leading to $^{15}N$ enrichment in seawater) in either sediment-dominated or sediment-impacted environments.

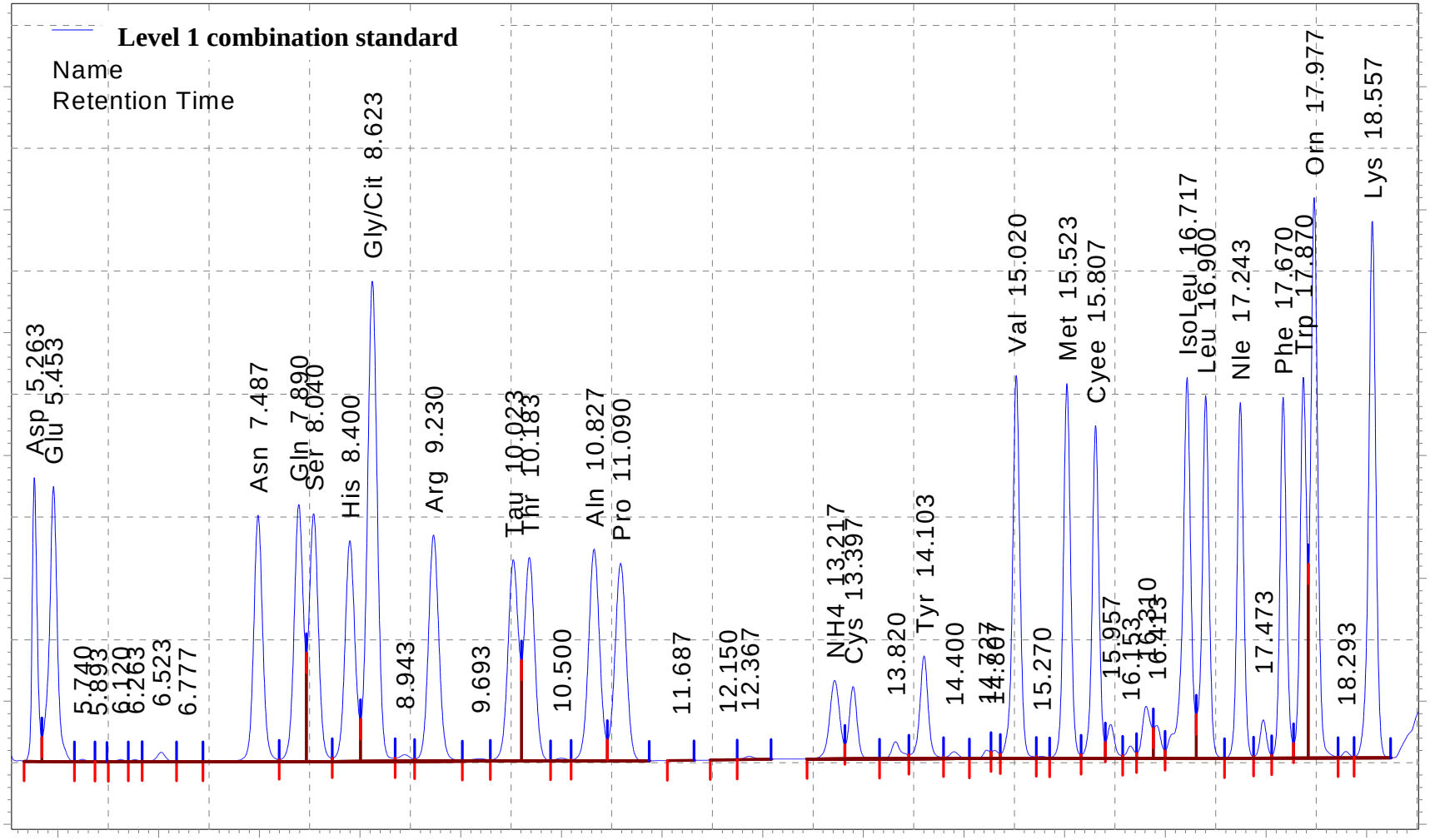
The mean $\delta^{15}N$ value for *Ulva* growing in all exposed rural sites around New Zealand, combined for summer and winter, is 7.7 ± 0.2 ‰. In a study of *Ulva* growing in proximity to a sewage discharge (before and after closure) at Moa Point, Wellington, $\delta^{15}N$ values in the range of 7 ‰ to 8 ‰ were identified as 'acceptable' indicating no further impact of sewage (Rogers, 2003). It is therefore suggested that in combination with the quantitative N-indices identified in this study, *Ulva* tissue $\delta^{15}N$ values in the range of 6.7 to 8.8 ‰ represent a useful baseline in investigations of potentially human-impacted environments, at least in New Zealand.

***Conclusions***

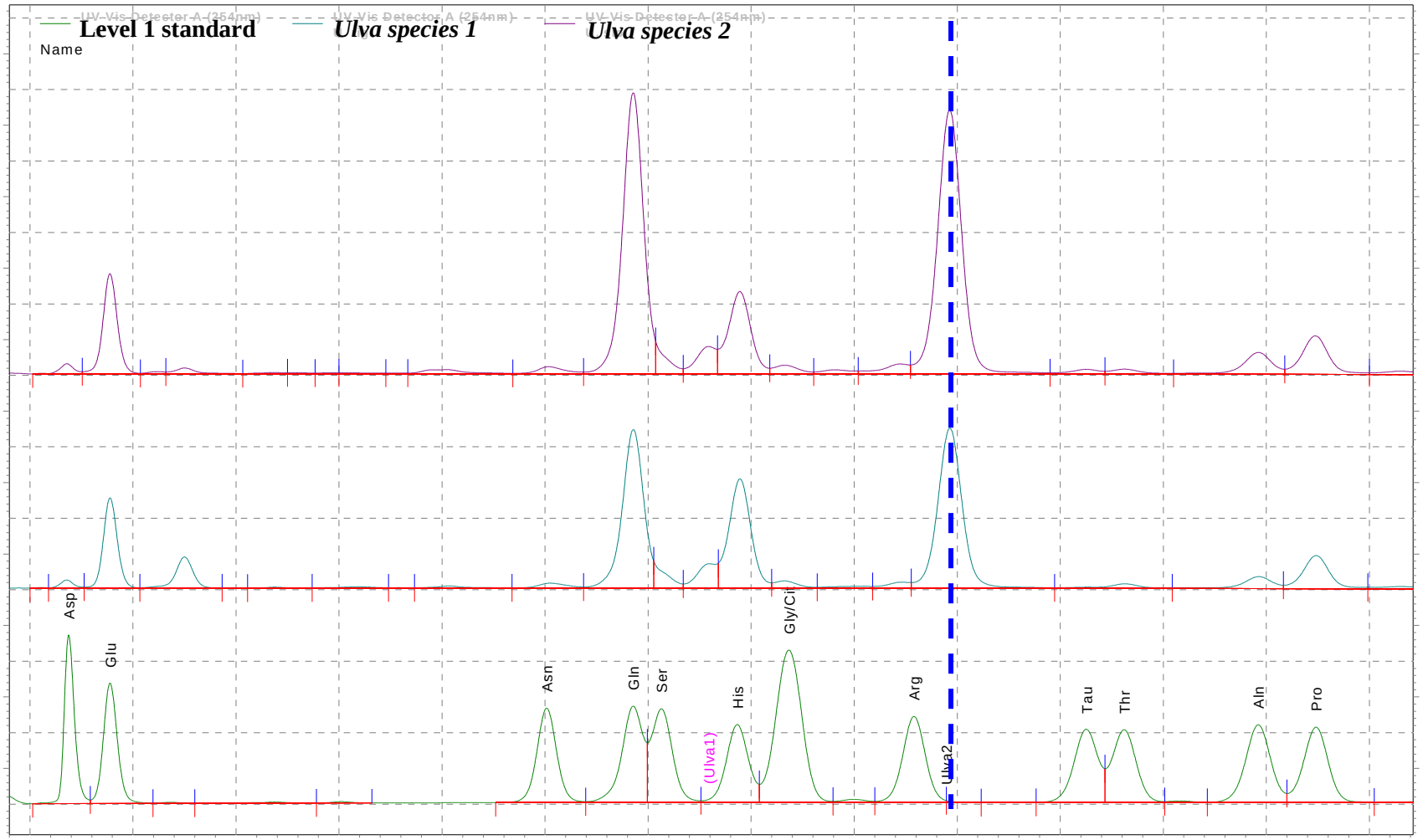
In summer, *Ulva* from environments with high TIN loading had correspondingly higher levels of free amino acids, particularly asparagine, and higher levels of chlorophyll and TN. In winter, *Ulva* populations on average contained significantly more nitrogen than their summertime counterparts, particularly those in colder seawater in more exposed coastal sites. However, these changes could not be explained by seasonal changes in seawater TIN concentrations alone. On the other hand TN content in *Ulva* from different environmental categories correlated well with seawater temperature, and to a lesser extent with surface irradiance, but there was a clear effect of nitrogen enrichment. It was concluded that several abiotic and biotic factors affect nitrogen status in natural populations of *Ulva*, but it is both the average nitrogen concentration in seawater, and the physical factors of temperature, light and water motion, that are mostly likely to be its overarching determinants.

**Appendix 2.1.** Details of dates and map grid coordinates of collection sites visited in summer and winter of 2002.

| Code | Location | Region | North/South Is. | Site description | Exposure | Urban / rural | Latitude | Longitude | Summer | Winter |
|---|---|---|---|---|---|---|---|---|---|---|
| **MP** | Mermaid Pools | Northland | North | high rock pool | sheltered | rural | 35°33' 32.63" S | 174°30' 52.37" E | 10/03/2002 | 8/10 /2002 |
| **MK** | Mokohinau Islands | Northland | North | offshore island | exposed | rural | 35°54' 27.29" S | 175°06' 25.99" E | 25/02/2002 | 2 3/08/2002 |
| **ON** | Onehunga lagoon | Auckland, west coast | North | sheltered tidal pond | sheltered | urban | 36°55' 27.71" S | 174°46' 35. 89" E | 17/03/2002 | 23/09/2002 |
| **WA** | Whangamata Estuary | Whangamata | North | sheltered harbour | sheltered | urban | 37°11' 45.31" S | 175°52' 14.78" E | 28/01 /2002 | 23/09/2002 |
| **MM** | Mount Manganui | Tauranga | North | moderatelty sheltered bay | exposed | urban | 37°37' 40.72" S | 176°10' 36.55" E | 3/01/ 2002 | not collected |
| **OT** | Otumoetai | Tauranga | North | large harbour | exposed | urban | 37° 39' 27.68" S | 176°08' 31.57" E | 3/01/2002 | 26/08/2002 |
| **HA** | Hardinge Road | Napier | North | sheltered embayment | exposed | urban | 39°03' 36.40" S | 174°02' 20.66" E | 24/03/2002 | 19/0 9/2002 |
| **HU** | Humber Road drain | Napier | North | sheltered embayment | sheltered | urban | 39°28' 43.04" S | 176°54' 01.88" E | 28/01/20 02 | 25/09/2002 |
| **NB** | Ngamutu Beach | New Plymouth | North | small harbour | sheltered | urban | 39°29' 13.90" S | 176°53' 14.86" E | 28/01/2002 | no t collected |
| **MA** | Makara Bay | Wellington | North | moderatelty sheltered bay | exposed | rural | 41°13' 10.37" S | 174°42' 38.08" E | 10/02/2 002 | 30/08/2002 |
| **FT** | Ferry Terminal | Wellington | North | large harbour | exposed | urban | 41°15' 43.62" S | 174°47' 17.95" E | 30/01/2002 | 29/08 /2002 |
| **EA** | Days Bay | Wellington | North | large harbour | exposed | urban | 41° 16' 58.89" S | 174°54' 16.48" E | 30/01/2002 | 28/08/2002 |
| **PA** | Point Arthur | Wellington | North | large harbour | exposed | urban | 41°18' 22.05" S | 174°53' 01.64" E | not collected | 28/0 8/2002 |
| **MO** | Moa Point discharge | Wellington | North | exposed embayment | exposed | urban | 41°20' 32.35" S | 174°48' 36.05" E | 29/01/ 2002 | 29/08/2002 |
| **FP** | Fossil Point | Farewell Spit | South | exposed rocky coast | exposed | rural | 40°30' 23.53" S | 172°43' 47.94" E | 1/02/200 2 | 6/09/2002 |
| **FW** | Farewell Bridge | Farewell Spit | South | channel, high tidal current | sheltered | rural | 40°31' 20.67" S | 172°44' 10. 72" E | not collected | 6/09/2002 |
| **IN** | Inner Beach | Farewell Spit | South | sheltered embayment | sheltered | rural | 40°31' 44.05" S | 172°45' 11.73" E | 1/02/20 02 | 6/09/2002 |
| **CA** | Cable Bay | Nelson | South | sheltered embayment | sheltered | rural | 41°08' 17.60" S | 173°01' 24.38" E | 2/02/2002 | 2/09/20 02 |
| **AM** | Amatel Wharf | Nelson | South | sheltered embayment | sheltered | urban | 41°08' 46.81" S | 173°00' 49.80" E | 31/01/2002 | 2/0 9/2002 |
| **CH** | Charlston rockpools, Constance Bay | West Coast | South | high rock pool | sheltered | rural | 41°10' 00.81" S | 173°26' 29.65" E | 8/02/2002 | 13/09/2002 |
| **TL** | Talleys Factory discharge | Motueka | South | sheltered embayment | sheltered | urban | 41°15' 14.41" S | 173°16' 56.16" E | 8/02/2002 | 14/09/2002 |
| **MT** | Motere Inlet | Motueka | South | sheltered embayment | sheltered | urban | 41°54' 07.41" S | 171°26' 03.07" E | 3/02/2002 | 7/0 9/2002 |
| **TM** | Tirimoana, Fox River mouth | West Coast | South | exposed rocky coast | exposed | rural | 42°01' 51.61" S | 171°22' 53.49 " E | 3/02/2002 | 7/09/2002 |
| **PI** | Paia Point | Kaikoura | South | exposed rocky coast | exposed | rural | 42°28' 20.91" S | 173°32' 10.87" E | 7/02/2002 | 13/09 /2002 |
| **ET** | Ebbtide Road, Avon-Heathcote Estuary | Christchurch | South | sheltered estuary | sheltered | urban | 43°32' 33.65" S | 17 2°44' 13.14" E | 6/02/2002 | 12/09/2002 |
| **MC** | McCormacks Bay, Avon Heathcote Estuary | Christchurch | South | sheltered estuary | sheltered | urban | 43°33' 23.68" S | 172°43' 25.06" E | not collected | 12/09/2002 |
| **SS** | South Spit Road, Avon-Heathcote Estuary | Christchurch | South | sheltered estuary | sheltered | urban | 43°33' 29.01" S | 172°44' 36.02" E | 6/02/2002 | not collected |
| **HO** | Hoopers Inlet | Dunedin | South | sheltered inlet | sheltered | rural | 45°51' 22.32" S | 170°40' 20.31" E | not collected | 11 /09/2002 |
| **TG** | Taggart Road, Dunedin Harbour | Dunedin | South | large harbour | exposed | urban | 45°51' 24.59" S | 170°35' 55.00" E | 5/0 2/2002 | 10/09/2002 |
| **SK** | Saint Kilda sewage discharge | Dunedin | South | exposed rocky coast | exposed | urban | 45°54' 31.83" S | 170°31' 56.14" E | not collected | 7/09/2002 |
| **BB** | Brighton beach | Dunedin | South | exposed rocky coast | exposed | rural | 45°56' 54.81" S | 170°20' 10.79" E | 5/02/2002 | 10/ 09/2002 |
| **TI** | Taieri | Dunedin | South | exposed estuary mouth | exposed | rural | 46°03 ' 06.10" S | 170°12' 20.23" E | 5/02/2002 | 10/09/2002 |




**Appendix 2.2.** Example chromatogram showing combination amino acid standards used to quantify amino acids in unknown samples.




**Appendix 2.3.** Example peak of unknown amino compound dominant in some *Ulva* (top two traces). Denoted as U2$^{9.48}$ and indicted by the dashed blue line in chromatogram below, it elutes just after arginine (Arg). Note the lower trace is a standard for reference.

**Appendix 2.4.** Seawater nutrient concentrations at coastal sites around New Zealand in summer 2002.

| Site | Code | Category | Temperature | Surface irradiance | TIN | | $NH_4^+$ | | $NO_2^-$ | | $NO_3^-$ | | $NH_4^+$/ TIN | $PO_4^{3-}$ | | N : P | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (℃) | (MJ · m$^{-2}$ · d$^{-1}$) | (µM) | | | | | | | | (%) | (µM) | | | |
| | | | | | mean | se | mean | se | mean | se | mean | se | mean | mean | se | mean | se |
| Cable Bay | CA | sheltered rural | 21.0 | 23.5 | 3.85 | 0.22 | 3.72 | 0.20 | 0.05 | 0.01 | 0.08 | 0.01 | 96.6 | 0.50 | 0.03 | 7.67 | 0.27 |
| Inner Bch | IN | sheltered rural | 21.0 | 21.9 | 0.53 | 0.07 | 0.22 | 0.04 | 0.08 | 0.00 | 0.22 | 0.03 | 42.6 | 0.68 | 0.04 | 0.79 | 0.06 |
| Brighton Beach | BB | exposed rural | 15.8 | 15.8 | 0.32 | 0.09 | 0.09 | 0.02 | 0.11 | 0.03 | 0.12 | 0.04 | 27.8 | 0.34 | 0.05 | 0.98 | 0.18 |
| Fossil Point | FP | exposed rural | 19.0 | 24.5 | 1.71 | 0.15 | 1.53 | 0.11 | 0.08 | 0.01 | 0.10 | 0.03 | 89.4 | 0.50 | 0.02 | 3.43 | 0.08 |
| Makara | MA | exposed rural | 16.8 | 22.3 | 7.55 | 0.90 | 6.13 | 0.70 | 0.34 | 0.02 | 1.08 | 0.18 | 81.2 | 1.20 | 0.04 | 6.32 | 0.66 |
| Mokohinau Islands | MK | exposed rural | 23.0 | 20.9 | 1.25 | 0.58 | 0.69 | 0.26 | 0.06 | 0.04 | 0.51 | 0.29 | 54.8 | 0.16 | 0.05 | 7.31 | 1.46 |
| Paia Point | PI | exposed rural | 16.0 | 18.4 | 13.80 | 0.33 | 3.14 | 0.15 | 0.31 | 0.01 | 10.35 | 0.17 | 22.8 | 1.53 | 0.03 | 9.05 | 0.33 |
| Taieri | TI | exposed rural | 15.5 | 15.8 | 1.39 | 0.11 | 1.13 | 0.04 | 0.16 | 0.01 | 0.11 | 0.05 | 81.0 | 0.48 | 0.02 | 2.90 | 0.26 |
| Tiromoana | TM | exposed rural | 20.0 | 23.5 | 2.23 | 1.09 | 1.99 | 1.01 | 0.14 | 0.00 | 0.10 | 0.08 | 89.3 | 0.31 | 0.02 | 7.11 | 2.89 |
| Charlston | CH | rock pool | 19.0 | 24.5 | 11.55 | 4.22 | 11.42 | 4.14 | 0.03 | 0.01 | 0.10 | 0.07 | 98.9 | 0.22 | 0.02 | 50.05 | 15.02 |
| Mermaid Pools | MP | rock pool | 25.0 | 18.3 | 3.44 | 0.18 | 2.75 | 0.13 | 0.06 | 0.01 | 0.63 | 0.04 | 79.8 | 0.11 | 0.02 | 35.22 | 7.07 |
| Amatel | AM | sheltered urban | 22.0 | 23.5 | 2.43 | 0.54 | 2.06 | 0.35 | 0.18 | 0.05 | 0.20 | 0.14 | 84.6 | 0.51 | 0.03 | 4.72 | 0.57 |
| Motere Inlet | MT | sheltered urban | 27.0 | 21.9 | 4.22 | 0.24 | 3.61 | 0.15 | 0.26 | 0.03 | 0.34 | 0.06 | 85.7 | 1.05 | 0.05 | 4.02 | 0.16 |
| Ngamutu Beach | NB | sheltered urban | 21.5 | 16.9 | 6.51 | 2.51 | 3.53 | 1.69 | 0.17 | 0.02 | 2.81 | 0.79 | 54.3 | 0.51 | 0.12 | 11.93 | 1.54 |
| Otumoetai | OT | sheltered urban | 22.0 | 24.8 | 7.27 | 4.70 | 2.38 | 0.00 | 0.02 | 0.02 | 4.86 | 4.68 | 32.8 | 0.34 | 0.05 | 27.21 | 20.26 |
| Whangamata | WA | sheltered urban | 22.0 | 22.9 | 1.05 | 0.10 | 0.65 | 0.01 | 0.12 | 0.03 | 0.28 | 0.05 | 61.5 | 0.15 | 0.03 | 7.76 | 1.47 |
| Eastbourne Beach | EA | exposed urban | 22.0 | 22.3 | 1.58 | 0.14 | 0.97 | 0.04 | 0.17 | 0.01 | 0.44 | 0.09 | 61.3 | 1.05 | 0.28 | 1.67 | 0.34 |
| Ferry Terminal | FT | exposed urban | 22.0 | 22.3 | 0.31 | 0.07 | 0.00 | 0.01 | 0.09 | 0.01 | 0.22 | 0.06 | 0.0 | 0.62 | 0.02 | 0.50 | 0.09 |
| Hardinge Road | HA | exposed urban | 20.0 | 23.2 | 0.10 | 0.31 | 0.00 | 0.17 | 0.00 | 0.01 | 0.00 | 0.14 | 0.0 | 0.07 | 0.04 | 2.23 | 0.16 |
| Mount Maunganganui | MM | exposed urban | 19.0 | 24.8 | 4.70 | 0.87 | 4.09 | 0.74 | 0.06 | 0.01 | 0.56 | 0.12 | 87.0 | 0.49 | 0.09 | 10.78 | 3.18 |
| Moa Point | MO | exposed urban | 19.3 | 22.3 | 0.22 | 0.17 | 0.11 | 0.02 | 0.04 | 0.01 | 0.07 | 0.15 | 52.0 | 0.35 | 0.01 | 0.63 | 0.45 |
| Taggart Road | TG | exposed urban | 15.8 | 17.2 | 1.17 | 0.18 | 0.77 | 0.12 | 0.15 | 0.01 | 0.25 | 0.05 | 66.0 | 0.68 | 0.01 | 1.73 | 0.24 |
| Ebbtide Street | ET | enriched urban | 17.5 | 17.1 | 109.16 | 2.16 | 92.23 | 1.72 | 2.71 | 0.02 | 14.21 | 0.41 | 84.5 | 3.43 | 0.92 | 39.74 | 14.70 |
| Humber Road | HU | enriched urban | 22.0 | 23.2 | 49.58 | 1.19 | 37.04 | 0.77 | 1.92 | 0.07 | 10.63 | 0.35 | 74.7 | 21.98 | 0.28 | 2.26 | 0.06 |
| Onehunga lagoon | ON | enriched urban | 21.5 | 16.8 | 10.31 | 0.38 | 5.17 | 0.09 | 1.10 | 0.01 | 4.04 | 0.28 | 50.2 | 6.68 | 0.12 | 1.55 | 0.07 |
| South Spit | SS | enriched urban | 17.0 | 17.1 | 13.12 | 0.60 | 12.90 | 0.46 | 0.22 | 0.05 | 0.00 | 0.09 | 98.3 | 1.09 | 0.13 | 12.22 | 1.14 |
| Talleys Discharge | TL | enriched urban | 20.0 | 21.9 | 9.06 | 0.25 | 6.52 | 0.17 | 0.22 | 0.02 | 2.32 | 0.06 | 72.0 | 1.72 | 0.10 | 5.29 | 0.20 |

**Appendix 2.5.** Nitrogen indices in *Ulva* at coastal sites around New Zealand in summer 2002. Note: asterisks for TN indicate analysis for single values for composite replicates.

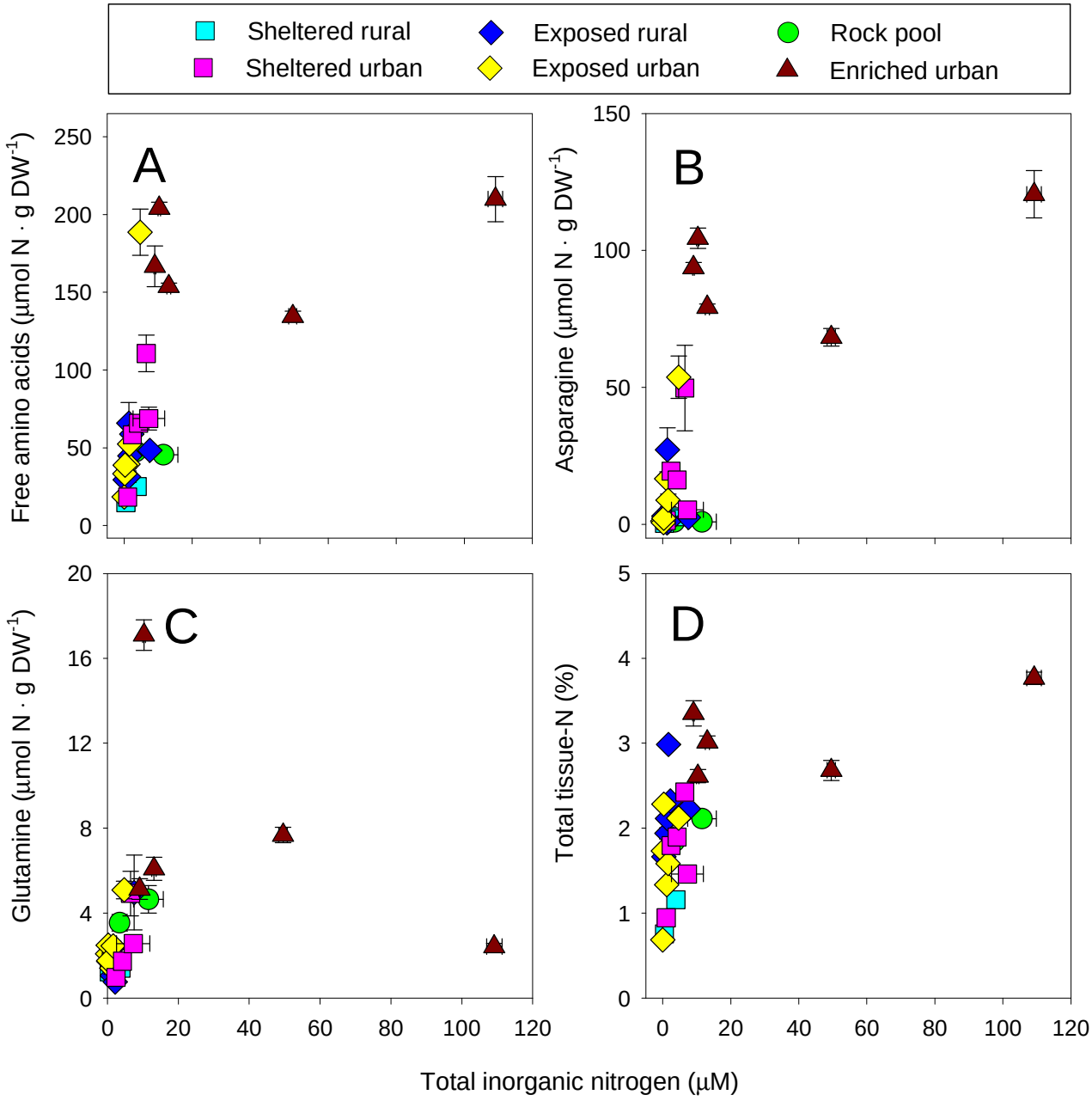
| Site | Code | Category | Total FAA | | Asn | | Asp | | Asn : Asp | Gln | | Glu | | Gln : Glu | His | | Pro | | U2$^{9.48}$ | | Other FAA | Chl a | | Chl b | | TChl | | FAA N / total N | | TN | | $\delta^{15}$N | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (μmol N · g DW$^{-1}$) | | | | | | | (μmol N · g DW$^{-1}$) | | | | | (μmol N · g DW$^{-1}$) | | | | | | | (μg · g DW$^{-1}$) | | | | | | (%) | | (%) | | (‰) | |
| | | | mean | se | mean | se | mean | se | mean | mean | se | mean | se | mean | mean | se | mean | se | mean | se | mean | mean | se | mean | se | mean | se | mean | se | mean | se | mean | se |
| Cable Bay | CA | sheltered rural | 25.1 | 3.8 | 2.7 | 0.9 | 0.5 | 0.1 | 5.5 | 1.4 | 0.2 | 2.4 | 0.3 | 0.6 | 9.2 | 1.1 | 1.3 | 0.6 | 0.8 | 0.2 | 6.8 | 1.1 | 0.1 | 0.7 | 0.1 | 1.8 | 0.1 | 3.0 | 0.5 | 1.2 | 0.0 | 7.2 | 0.3 |
| Inner Bch | IN | sheltered rural | 14.6 | 1.3 | 0.4 | 0.0 | 0.6 | 0.1 | 0.6 | 1.2 | 0.4 | 2.2 | 0.8 | 0.6 | 5.1 | 0.5 | 0.3 | 0.1 | 0.6 | 0.0 | 4.3 | 0.7 | 0.0 | 0.4 | 0.1 | 1.1 | 0.1 | 2.7 | 0.2 | 0.8 | 0.0 | 7.7 | 0.5 |
| Brighton Beach | BB | exposed rural | 29.4 | 0.7 | 3.0 | 0.3 | 0.9 | 0.0 | 3.4 | 1.7 | 0.2 | 1.6 | 0.2 | 1.1 | 13.2 | 0.6 | 2.6 | 0.9 | 0.7 | 0.1 | 5.6 | 0.8 | 0.1 | 0.7 | 0.0 | 1.5 | 0.1 | 2.5 | 0.1 | 1.7 | * | 8.2 | * |
| Fossil Point | FP | exposed rural | 44.9 | 1.5 | 1.0 | 0.1 | 0.8 | 0.0 | 1.3 | 1.9 | 0.2 | 2.3 | 0.2 | 0.8 | 29.7 | 1.2 | 1.3 | 0.2 | 1.8 | 0.5 | 6.2 | 2.6 | 0.2 | 1.7 | 0.1 | 4.3 | 0.3 | 2.1 | 0.1 | 3.0 | * | 7.6 | * |
| Makara | MA | exposed rural | 48.4 | 0.3 | 2.5 | 1.1 | 0.9 | 0.0 | 2.9 | 5.0 | 1.8 | 5.2 | 0.2 | 1.0 | 23.7 | 0.3 | 2.9 | 0.5 | 1.4 | 0.6 | 6.9 | 1.7 | 0.1 | 1.2 | 0.1 | 2.9 | 0.2 | 3.0 | 0.0 | 2.2 | * | 7.9 | * |
| Mokohinau Islands | MK | exposed rural | 31.4 | 3.6 | 0.6 | 0.1 | 0.5 | 0.0 | 1.1 | 2.3 | 0.2 | 2.5 | 0.6 | 0.9 | 17.6 | 2.2 | 0.4 | 0.1 | 0.8 | 0.4 | 6.6 | 3.3 | 0.1 | 2.1 | 0.1 | 5.4 | 0.2 | 2.3 | 0.3 | 1.9 | 0.1 | 6.7 | 0.1 |
| Paia Point | PI | exposed rural | | | | | | | | | | | | | | | | | | | | 2.4 | 0.2 | 2.2 | 0.1 | 4.6 | 0.3 | | | 2.7 | * | 8.8 | * |
| Taieri | TI | exposed rural | 65.8 | 13.4 | 27.2 | 8.0 | 1.1 | 0.1 | 25.1 | 1.1 | 0.2 | 2.0 | 0.4 | 0.5 | 25.2 | 3.5 | 2.1 | 1.0 | 0.9 | 0.1 | 6.4 | 2.3 | 0.1 | 1.9 | 0.1 | 4.3 | 0.2 | 4.4 | 0.9 | 2.1 | * | 7.9 | * |
| Tiromoana | TM | exposed rural | 58.9 | 2.3 | 17.8 | 1.0 | 1.0 | 0.1 | 18.0 | 0.8 | 0.0 | 3.4 | 0.2 | 0.2 | 27.1 | 1.4 | 3.7 | 0.8 | 0.6 | 0.0 | 4.4 | 2.1 | 0.2 | 2.1 | 0.2 | 4.2 | 0.4 | 3.6 | 0.1 | 2.3 | * | 7.7 | * |
| Charlston | CH | rock pool | 45.5 | 2.0 | 0.9 | 0.0 | 1.2 | 0.1 | 0.7 | 4.6 | 0.6 | 4.2 | 0.2 | 1.1 | 24.2 | 1.0 | 2.6 | 0.3 | 1.7 | 0.2 | 6.1 | 2.7 | 0.1 | 1.6 | 0.1 | 4.3 | 0.2 | 3.0 | 0.1 | 2.1 | * | 7.4 | * |
| Mermaid Pools | MP | rock pool | 47.5 | 3.3 | 0.9 | 0.2 | 1.0 | 0.0 | 0.9 | 3.5 | 0.4 | 3.3 | 0.2 | 1.1 | 26.0 | 1.6 | 1.6 | 0.2 | 2.4 | 0.7 | 8.9 | 2.9 | 0.1 | 1.8 | 0.1 | 4.7 | 0.3 | 3.6 | 0.3 | 1.8 | * | 6.6 | * |
| Amatel | AM | sheltered urban | 58.3 | 1.4 | 19.4 | 1.5 | 1.2 | 0.0 | 15.7 | 1.0 | 0.0 | 3.7 | 0.1 | 0.3 | 23.7 | 0.5 | 1.5 | 0.0 | 1.3 | 0.1 | 6.4 | 1.8 | 0.1 | 1.4 | 0.2 | 3.2 | 0.3 | 4.5 | 0.1 | 1.8 | * | 9.5 | * |
| Motere Inlet | MT | sheltered urban | 65.8 | 2.9 | 16.2 | 1.6 | 1.4 | 0.1 | 11.4 | 1.7 | 0.2 | 5.0 | 0.2 | 0.3 | 27.5 | 0.8 | 3.4 | 0.6 | 1.4 | 0.1 | 9.1 | 2.7 | 0.2 | 2.1 | 0.1 | 4.8 | 0.3 | 4.9 | 0.2 | 1.9 | * | 8.9 | * |
| Ngamutu Beach | NB | sheltered urban | 110.7 | 11.8 | 49.7 | 15.6 | 1.6 | 0.2 | 32.1 | 4.9 | 1.0 | 6.5 | 0.7 | 0.8 | 28.8 | 1.5 | 10.0 | 2.9 | 2.7 | 0.7 | 6.5 | 3.4 | 0.2 | 2.9 | 0.1 | 6.4 | 0.1 | 6.4 | 0.7 | 2.4 | * | 7.3 | * |
| Otumoetai | OT | sheltered urban | 68.8 | 7.4 | 5.3 | 1.0 | 2.0 | 0.6 | 2.6 | 2.6 | 0.2 | 8.8 | 0.8 | 0.3 | 36.6 | 3.9 | 3.0 | 0.2 | 0.8 | 0.1 | 9.6 | 1.6 | 0.0 | 1.4 | 0.0 | 3.0 | 0.1 | 6.6 | 0.7 | 1.5 | 0.0 | 7.1 | 0.1 |
| Whangamata | WA | sheltered urban | 18.3 | 1.0 | 1.4 | 0.1 | 0.4 | 0.0 | 3.1 | 1.7 | 0.1 | 2.1 | 0.2 | 0.8 | 7.2 | 0.3 | 0.6 | 0.0 | 0.6 | 0.0 | 4.2 | 0.6 | 0.0 | 0.4 | 0.0 | 1.0 | 0.1 | 2.7 | 0.1 | 0.9 | * | 8.5 | * |
| Eastbourne Beach | EA | exposed urban | 52.4 | 2.3 | 8.8 | 2.3 | 1.1 | 0.1 | 8.0 | 2.5 | 0.2 | 4.9 | 0.2 | 0.5 | 22.8 | 1.6 | 3.7 | 1.2 | 1.0 | 0.1 | 7.7 | 1.5 | 0.1 | 1.0 | 0.1 | 2.5 | 0.2 | 4.6 | 0.2 | 1.6 | * | 10.7 | * |
| Ferry Terminal | FT | exposed urban | 38.9 | 2.4 | 2.2 | 0.2 | 0.8 | 0.1 | 2.7 | 1.8 | 0.2 | 3.4 | 0.6 | 0.5 | 18.7 | 1.0 | 5.8 | 0.3 | 0.5 | 0.0 | 5.7 | 2.1 | 0.1 | 2.0 | 0.3 | 4.0 | 0.3 | 2.4 | 0.1 | 2.3 | * | 7.9 | * |
| Hardinge Road | HA | exposed urban | 18.4 | 0.2 | 1.1 | 0.2 | 0.5 | 0.1 | 2.4 | 2.1 | 0.1 | 1.2 | 0.1 | 1.7 | 7.0 | 0.2 | 1.1 | 0.3 | 0.2 | 0.0 | 5.2 | 0.6 | 0.0 | 0.5 | 0.1 | 1.1 | 0.1 | 3.8 | 0.0 | 0.7 | 0.0 | 8.8 | 0.0 |
| Mount Maunganganui | MM | exposed urban | 188.6 | 14.9 | 53.7 | 7.7 | 2.9 | 0.4 | 18.2 | 5.1 | 0.4 | 10.4 | 0.7 | 0.5 | 71.5 | 2.8 | 19.1 | 2.4 | 15.9 | 3.5 | 10.0 | 3.1 | 0.2 | 2.0 | 0.2 | 5.1 | 0.4 | 12.5 | 1.0 | 2.1 | * | 6.9 | * |
| Moa Point | MO | exposed urban | 33.3 | 1.7 | 0.6 | 0.1 | 0.9 | 0.1 | 0.6 | 2.5 | 0.1 | 4.3 | 0.0 | 0.6 | 16.3 | 1.1 | 0.5 | 0.1 | 0.5 | 0.1 | 7.7 | 2.2 | 0.2 | 1.8 | 0.4 | 4.0 | 0.6 | 2.7 | 0.1 | 1.7 | * | 8.2 | * |
| Taggart Road | TG | exposed urban | 39.4 | 2.9 | 16.6 | 0.5 | 0.8 | 0.1 | 20.2 | 1.4 | 0.3 | 2.3 | 0.4 | 0.6 | 11.0 | 1.1 | 1.1 | 0.5 | 1.2 | 0.1 | 4.9 | 1.0 | 0.1 | 1.2 | 0.2 | 2.2 | 0.3 | 4.1 | 0.3 | 1.3 | * | 9.4 | * |
| Ebbtide Street | ET | enriched urban | 209.9 | 14.5 | 120.4 | 8.6 | 2.3 | 0.1 | 51.4 | 2.4 | 0.2 | 4.9 | 0.5 | 0.5 | 33.9 | 1.5 | 29.8 | 2.7 | 9.5 | 0.8 | 6.5 | 3.5 | 0.1 | 3.3 | 0.2 | 6.8 | 0.3 | 7.8 | 0.5 | 3.8 | 0.1 | 10.0 | 0.5 |
| Humber Road | HU | enriched urban | 134.4 | 3.6 | 68.3 | 3.2 | 2.1 | 0.1 | 33.0 | 7.7 | 0.4 | 3.8 | 0.1 | 2.0 | 29.2 | 1.1 | 2.6 | 0.3 | 9.6 | 0.7 | 11.2 | 2.5 | 0.1 | 2.4 | 0.1 | 4.9 | 0.2 | 7.0 | 0.2 | 2.7 | 0.1 | 8.2 | 0.1 |
| Onehunga lagoon | ON | enriched urban | 204.0 | 3.8 | 104.4 | 3.8 | 2.6 | 0.2 | 39.9 | 17.1 | 0.7 | 12.9 | 0.8 | 1.3 | 45.6 | 1.2 | 1.6 | 0.3 | 1.8 | 0.1 | 17.9 | 3.5 | 0.1 | 9.7 | 0.6 | 13.2 | 0.6 | 10.9 | 0.2 | 2.6 | 0.1 | 15.1 | 0.1 |
| South Spit | SS | enriched urban | 153.8 | 2.1 | 79.3 | 1.1 | 2.6 | 0.1 | 30.4 | 6.1 | 0.5 | 4.6 | 0.3 | 1.3 | 34.4 | 0.6 | 9.2 | 1.5 | 6.2 | 0.6 | 11.5 | 2.8 | 0.2 | 2.5 | 0.2 | 5.3 | 0.4 | 7.1 | 0.1 | 3.0 | 0.1 | 11.9 | 0.3 |
| Talleys Discharge | TL | enriched urban | 166.7 | 13.0 | 93.7 | 1.8 | 3.1 | 0.1 | 30.5 | 5.1 | 0.5 | 5.5 | 1.7 | 0.9 | 34.2 | 2.7 | 10.1 | 5.4 | 4.7 | 0.3 | 10.3 | 3.1 | 0.1 | 3.1 | 0.1 | 6.1 | 0.2 | 7.0 | 0.5 | 3.4 | 0.1 | 6.0 | 0.1 |

**Appendix 2.6.** Seawater nutrient concentrations at coastal sites around New Zealand in winter 2002.

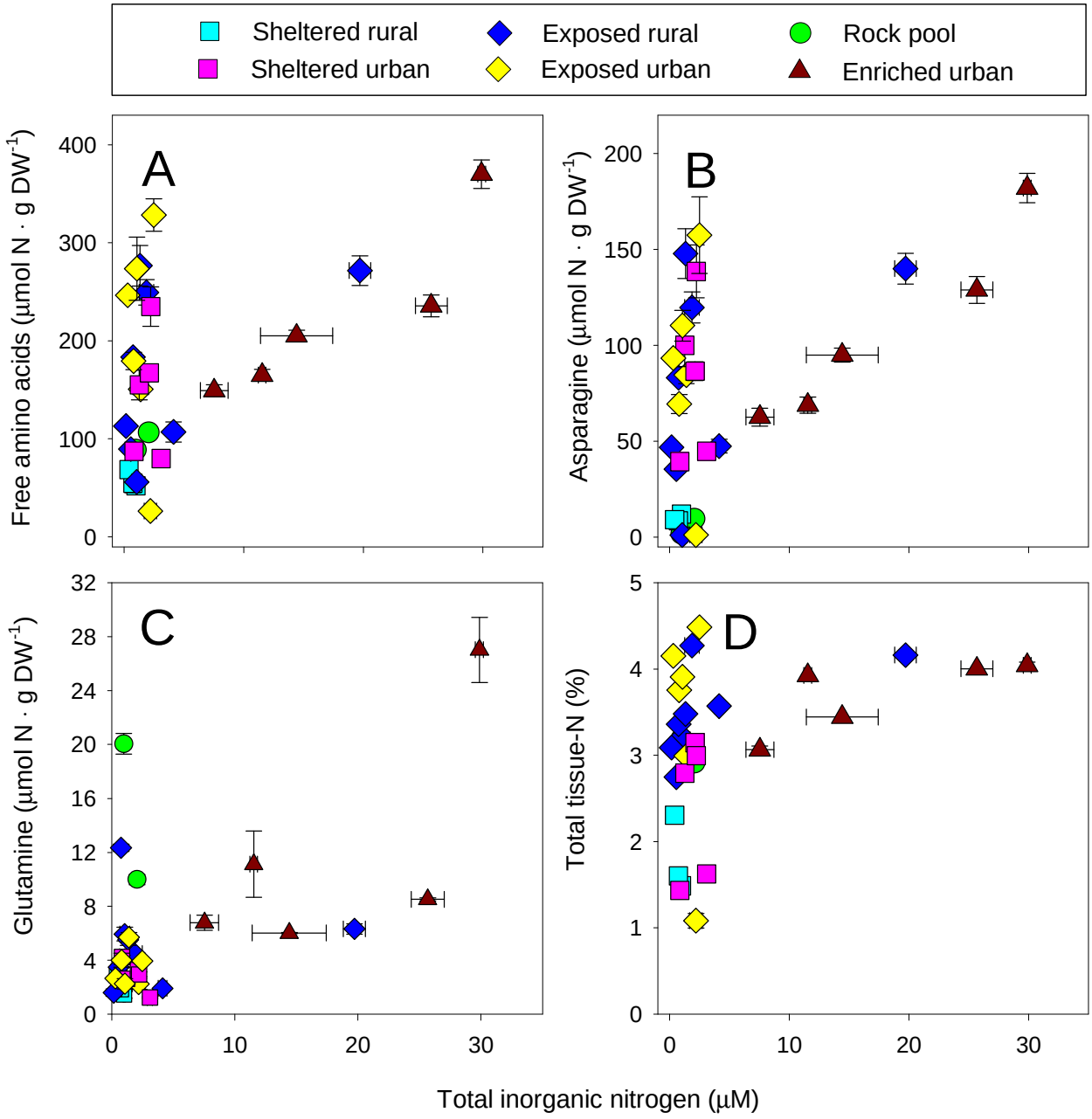
| Site | Code | Category | Temperature | Surface irradiance | TIN | | $NH_4^+$ | | $NO_2^-$ | | $NO_3^-$ | | $NH_4^+$/ TIN | $PO_4^{3-}$ | | N : P | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (℃) | (MJ · $m^{-2}$ · $d^{-1}$) | (µM) | | | | | | | | (%) | (µM) | | | |
| | | | | | mean | se | mean | se | mean | se | mean | se | mean | mean | se | mean | se |
| Cable Bay | CA | sheltered urban | 12.5 | 12.0 | 0.41 | 0.02 | 0.30 | 0.04 | 0.04 | 0.02 | 0.06 | 0.06 | 73.6 | 0.34 | 0.05 | 1.26 | 0.24 |
| Hoopers Inlet | HO | sheltered urban | 13.0 | 7.4 | 1.00 | 0.07 | 0.93 | 0.08 | 0.14 | 0.01 | 0.00 | 0.01 | 93.4 | 0.49 | 0.16 | 2.67 | 0.98 |
| Inner Beach | IN | sheltered urban | 20.0 | 10.0 | 0.73 | 0.05 | 0.52 | 0.04 | 0.06 | 0.02 | 0.15 | 0.02 | 71.1 | 0.56 | 0.02 | 1.32 | 0.12 |
| Brighton Beach | BB | exposed rural | 10.8 | 7.8 | 1.32 | 0.15 | 0.25 | 0.03 | 0.27 | 0.08 | 0.80 | 0.18 | 18.7 | 0.35 | 0.15 | 5.22 | 1.74 |
| Fossil Point | FP | exposed rural | 14.3 | 6.2 | 4.12 | 0.35 | 0.46 | 0.11 | 0.48 | 0.01 | 3.18 | 0.25 | 11.2 | 0.64 | 0.16 | 7.54 | 2.40 |
| Farewell Bridge | FW | exposed rural | 14.5 | 10.0 | 0.57 | 0.04 | 0.44 | 0.01 | 0.01 | 0.00 | 0.12 | 0.04 | 77.0 | 0.36 | 0.02 | 1.58 | 0.19 |
| Makara | MA | exposed rural | 13.0 | 8.6 | 19.71 | 0.90 | 0.60 | 0.09 | 0.42 | 0.02 | 18.70 | 0.81 | 3.0 | 0.22 | 0.02 | 92.83 | 12.44 |
| Mokohinau Islands | MK | exposed rural | 16.0 | 10.3 | 1.05 | 0.39 | 0.61 | 0.15 | 0.16 | 0.14 | 0.28 | 0.11 | 58.2 | 0.03 | 0.02 | 54.49 | 13.17 |
| Paia Point | PI | exposed rural | 9.8 | 10.5 | 0.75 | 0.09 | 0.60 | 0.06 | 0.12 | 0.02 | 0.03 | 0.07 | 80.3 | 0.09 | 0.01 | 8.42 | 1.66 |
| Taieri | TI | exposed rural | 10.8 | 7.8 | 1.86 | 0.60 | 0.72 | 0.16 | 0.24 | 0.02 | 0.91 | 0.44 | 38.5 | 0.79 | 0.13 | 2.34 | 0.74 |
| Tiromoana | TM | exposed rural | 12.5 | 8.6 | 0.15 | 0.09 | 0.00 | 0.07 | 0.16 | 0.01 | 0.01 | 0.03 | 0.0 | 0.17 | 0.02 | 0.91 | 0.48 |
| Charlston | CH | rock pool | 12.5 | 6.2 | 0.98 | 0.19 | 0.74 | 0.16 | 0.21 | 0.04 | 0.03 | 0.12 | 76.1 | 0.18 | 0.02 | 5.40 | 0.66 |
| Mermaid Pools | MP | rock pool | 16.0 | 14.2 | 2.05 | 0.18 | 1.46 | 0.09 | 0.16 | 0.02 | 0.43 | 0.08 | 71.3 | 0.38 | 0.03 | 5.43 | 0.43 |
| Amatel wharf | AM | sheltered urban | 12.8 | 12.0 | 1.26 | 0.02 | 0.84 | 0.03 | 0.11 | 0.03 | 0.30 | 0.06 | 67.2 | 0.52 | 0.07 | 2.52 | 0.35 |
| Motere Inlet | MT | sheltered urban | 11.8 | 10.0 | 2.22 | 0.10 | 0.72 | 0.01 | 0.24 | 0.02 | 1.26 | 0.12 | 32.6 | 0.49 | 0.04 | 4.64 | 0.60 |
| Ngamutu Beach | NB | sheltered urban | 15.5 | 11.4 | 2.12 | 0.20 | 0.35 | 0.09 | 0.45 | 0.01 | 1.32 | 0.10 | 16.4 | 0.33 | 0.04 | 6.52 | 0.58 |
| Otumoetai | OT | sheltered urban | 15.5 | 9.7 | 0.84 | 0.19 | 0.48 | 0.16 | 0.09 | 0.00 | 0.27 | 0.04 | 57.1 | 0.31 | 0.05 | 2.64 | 0.33 |
| Whangamata | WA | sheltered urban | 17.5 | 13.7 | 3.10 | 0.20 | 1.66 | 0.04 | 0.14 | 0.00 | 1.30 | 0.16 | 53.5 | 0.54 | 0.03 | 5.74 | 0.36 |
| Days Bay | EA | exposed urban | 13.0 | 8.6 | 0.31 | 0.12 | 0.14 | 0.02 | 0.06 | 0.01 | 0.11 | 0.13 | 44.6 | 0.17 | 0.03 | 1.91 | 0.90 |
| Ferry Terminal | FT | exposed urban | 11.3 | 8.6 | 2.48 | 0.22 | 0.28 | 0.05 | 0.21 | 0.06 | 1.99 | 0.12 | 11.3 | 0.48 | 0.11 | 5.62 | 1.23 |
| Hardinge Road | HA | exposed urban | 15.0 | 9.2 | 2.19 | 0.52 | 1.86 | 0.46 | 0.14 | 0.01 | 0.19 | 0.06 | 85.1 | 0.33 | 0.02 | 6.58 | 1.34 |
| Moa Point | MO | exposed urban | 11.5 | 8.6 | 0.80 | 0.08 | 0.11 | 0.09 | 0.25 | 0.02 | 0.43 | 0.04 | 14.3 | 0.24 | 0.05 | 3.65 | 1.00 |
| Point Arther | PA | exposed urban | 12.0 | 8.6 | 1.06 | 0.07 | 0.23 | 0.04 | 0.31 | 0.07 | 0.51 | 0.08 | 22.1 | 0.35 | 0.03 | 3.03 | 0.15 |
| Tagg Road | TG | exposed urban | 13.5 | 7.4 | 1.38 | 0.02 | 0.79 | 0.04 | 0.26 | 0.02 | 0.34 | 0.03 | 57.2 | 0.72 | 0.06 | 1.95 | 0.18 |
| Ebbtide Street | ET | enriched urban | 13.8 | 10.5 | 29.88 | 0.33 | 22.70 | 0.26 | 1.15 | 0.09 | 6.02 | 0.14 | 76.0 | 2.57 | 0.07 | 11.66 | 0.43 |
| McCormack's Bay | MC | enriched urban | 12.5 | 10.5 | 14.42 | 3.01 | 4.50 | 2.20 | 0.18 | 0.02 | 9.74 | 1.30 | 31.2 | 1.39 | 0.08 | 10.29 | 1.92 |
| Onehunga lagoon | ON | enriched urban | 15.0 | 12.2 | 7.53 | 1.16 | 5.92 | 0.99 | 0.20 | 0.01 | 1.41 | 0.17 | 78.6 | 6.07 | 0.18 | 1.23 | 0.12 |
| Saint Kilda discharge | SK | enriched urban | 10.5 | 7.4 | 11.54 | 0.31 | 11.05 | 0.19 | 0.26 | 0.01 | 0.23 | 0.22 | 95.8 | 1.10 | 0.01 | 10.47 | 0.20 |
| Talleys Factory | TL | enriched urban | 11.5 | 10.0 | 25.67 | 1.33 | 20.59 | 0.99 | 0.52 | 0.16 | 4.56 | 0.32 | 80.2 | 5.25 | 0.23 | 4.90 | 0.26 |

**Appendix 2.7.** Nitrogen indices in *Ulva* at coastal sites around New Zealand in winter 2002. Note: asterisks for TN indicate analysis for single values for composite replicates.

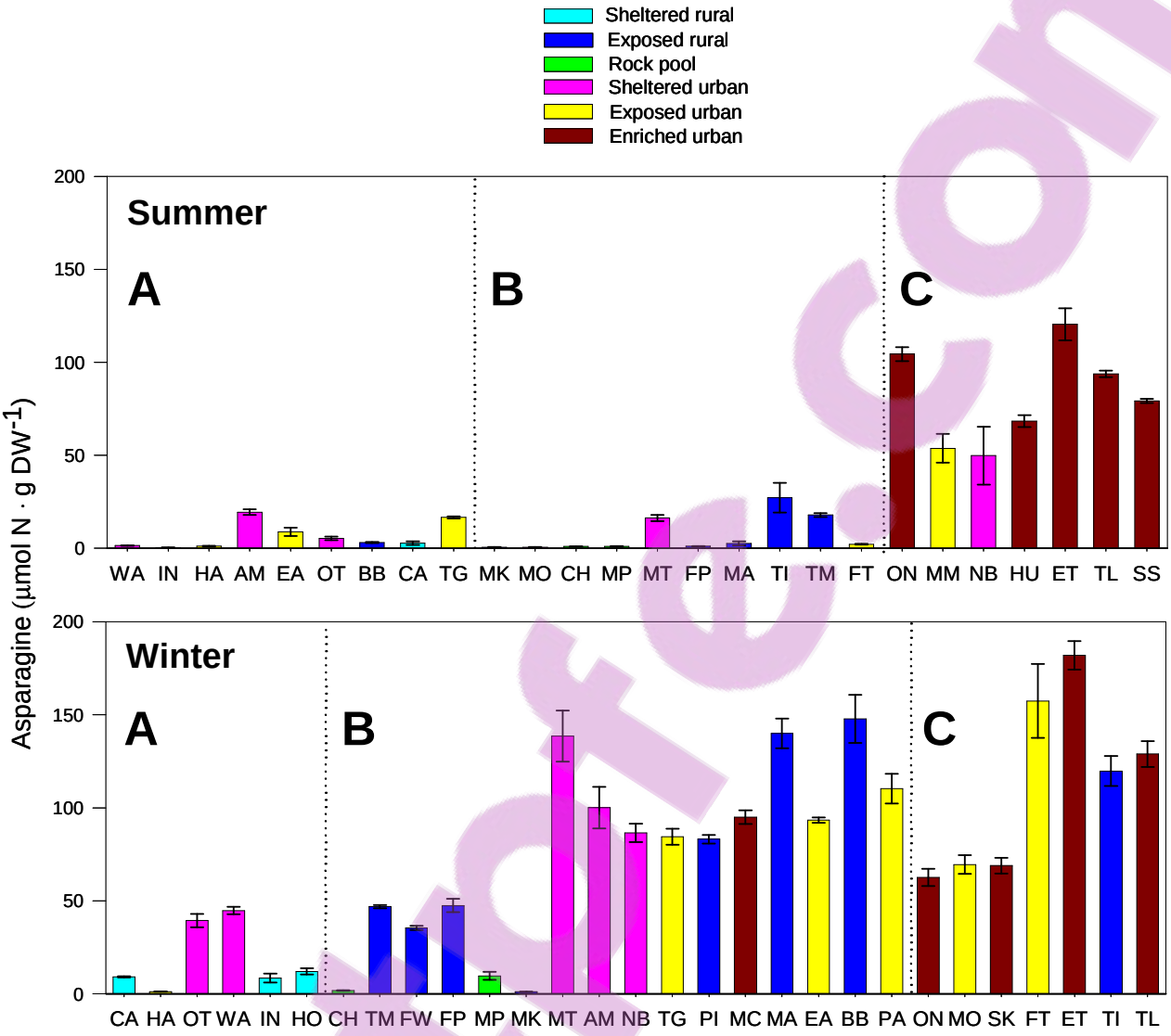
| Site | Code | Category | Total FAA | | Asn | | Asp | | Asn : Asp | Gln | | Glu | | Gln : Glu | His | | Pro | | U2$^{9.48}$ | | Other FAA | Chl a | | Chl b | | TChl | | FAA N / total N | | TN | | $\delta^{15}$N | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (μmol N · g DW$^{-1}$) | | | | | | | (μmol N · g DW$^{-1}$) | | | | | (μmol N · g DW$^{-1}$) | | | | | | | (μg · g DW$^{-1}$) | | | | | | (%) | | (%) | | (‰) | |
| | | | mean | se | mean | se | mean | se | mean | mean | se | mean | se | mean | mean | se | mean | se | mean | se | mean | mean | se | mean | se | mean | se | mean | se | mean | se | mean | se |
| Cable Bay | CA | sheltered urban | 68.7 | 1.7 | 9.1 | 0.3 | 0.9 | 0.1 | 10.1 | 2.9 | 0.1 | 7.0 | 0.4 | 0.4 | 19.9 | 0.4 | 18.5 | 0.8 | 2.3 | 0.4 | 8.1 | 2.3 | 0.1 | 1.5 | 0.0 | 3.8 | 0.1 | 4.2 | 0.1 | 2.3 | 0.0 | 6.2 | 0.0 |
| Hoopers Inlet | HO | sheltered urban | 52.1 | 4.4 | 12.0 | 1.7 | 1.1 | 0.1 | 10.5 | 1.5 | 0.3 | 9.4 | 0.6 | 0.2 | 16.3 | 1.3 | 2.9 | 0.5 | 0.4 | 0.1 | 8.6 | 1.9 | 0.0 | 1.6 | 0.0 | 3.5 | 0.1 | 4.9 | 0.4 | 1.5 | * | 8.0 | * |
| Inner Beach | IN | sheltered urban | 54.8 | 9.5 | 8.5 | 2.4 | 0.5 | 0.0 | 17.2 | 1.8 | 0.0 | 5.2 | 0.8 | 0.4 | 17.2 | 1.9 | 13.8 | 4.6 | 0.5 | 0.2 | 7.3 | 2.1 | 0.1 | 1.3 | 0.1 | 3.4 | 0.1 | 4.8 | 0.8 | 1.6 | 0.0 | 8.8 | 0.0 |
| Brighton Beach | BB | exposed rural | 276.5 | 20.6 | 147.8 | 13.0 | 2.7 | 0.2 | 55.4 | 5.5 | 0.4 | 12.8 | 1.2 | 0.4 | 62.2 | 4.2 | 32.1 | 1.0 | 2.0 | 0.5 | 11.5 | 3.4 | 0.1 | 2.5 | 0.1 | 6.0 | 0.2 | 11.1 | 0.8 | 3.5 | * | 8.0 | * |
| Fossil Point | FP | exposed rural | 107.0 | 10.2 | 47.4 | 3.6 | 0.8 | 0.1 | | 1.9 | 0.0 | 6.0 | 1.1 | | 33.5 | 3.0 | 9.7 | 2.7 | 1.9 | 0.1 | 5.8 | 2.9 | 0.1 | 2.3 | 0.0 | 5.2 | 0.2 | 4.2 | 0.4 | 3.6 | * | 7.6 | * |
| Farewell Bridge | FW | exposed rural | 89.5 | 3.4 | 35.5 | 1.1 | 1.3 | 0.0 | 27.6 | 3.5 | 0.2 | 8.6 | 0.4 | 0.4 | 24.5 | 1.4 | 5.4 | 0.7 | 1.4 | 0.2 | 9.3 | 3.6 | 0.3 | 2.9 | 0.2 | 6.5 | 0.4 | 4.6 | 0.2 | 2.7 | * | 7.9 | * |
| Makara | MA | exposed rural | 271.6 | 15.1 | 140.0 | 8.0 | 3.5 | 0.3 | 40.2 | 6.3 | 0.4 | 17.6 | 1.3 | 0.4 | 48.1 | 4.0 | 33.4 | 0.6 | 10.3 | 0.9 | 12.3 | 2.7 | 0.0 | 1.9 | 0.1 | 4.5 | 0.1 | 9.1 | 0.5 | 4.2 | * | 7.6 | * |
| Mokohinau Islands | MK | exposed rural | 56.0 | 4.3 | 1.0 | 0.1 | 0.9 | 0.0 | 1.1 | 5.9 | 0.5 | 4.0 | 0.2 | 1.5 | 26.5 | 2.7 | 4.9 | 0.7 | 3.0 | 0.5 | 9.6 | 3.0 | 0.0 | 1.7 | 0.0 | 4.7 | 0.0 | 2.4 | 0.2 | 3.2 | 0.1 | 7.1 | 0.0 |
| Paia Point | PI | exposed rural | 183.4 | 3.9 | 83.1 | 2.3 | 2.4 | 0.1 | 34.8 | 12.3 | 0.3 | 13.4 | 1.0 | 0.9 | 44.8 | 2.2 | 21.5 | 6.6 | 2.7 | 0.2 | 3.1 | 4.2 | 0.1 | 3.6 | 0.2 | 7.8 | 0.3 | 7.6 | 0.2 | 3.4 | * | 8.5 | * |
| Taieri | TI | exposed rural | 249.3 | 13.0 | 119.8 | 8.1 | 2.5 | 0.2 | 47.4 | 4.5 | 0.2 | 18.8 | 1.8 | 0.2 | 42.6 | 1.0 | 44.5 | 1.6 | 2.5 | 0.3 | 14.1 | 5.3 | 0.1 | 4.2 | 0.0 | 9.5 | 0.1 | 8.2 | 0.4 | 4.3 | * | 7.4 | * |
| Tiromoana | TM | exposed rural | 112.9 | 3.4 | 46.9 | 0.8 | 1.4 | 0.1 | 34.4 | 1.6 | 0.3 | 6.9 | 1.0 | 0.2 | 42.6 | 2.7 | 7.2 | 0.9 | 0.4 | 0.1 | 5.9 | 3.8 | 0.1 | 3.2 | 0.1 | 7.0 | 0.1 | 5.1 | 0.2 | 3.1 | * | 6.7 | * |
| Charlston | CH | rock pool | 88.6 | 1.1 | 1.7 | 0.2 | 1.0 | 0.1 | 1.8 | 20.0 | 0.8 | 8.0 | 0.2 | 2.5 | 31.9 | 0.5 | 12.8 | 1.4 | 5.4 | 0.5 | 7.8 | 4.5 | 0.1 | 2.6 | 0.0 | 7.1 | 0.1 | 3.9 | 0.0 | 3.2 | * | 7.0 | * |
| Mermaid Pools | MP | rock pool | 106.4 | 6.1 | 9.6 | 2.2 | 1.0 | 0.1 | 9.2 | 10.0 | 0.4 | 8.2 | 0.7 | 1.2 | 34.5 | 2.7 | 20.8 | 2.0 | 9.4 | 1.0 | 12.9 | 3.3 | 0.1 | 2.1 | 0.0 | 5.5 | 0.1 | 5.1 | 0.3 | 2.9 | * | 7.9 | * |
| Amatel wharf | AM | sheltered urban | 155.0 | 15.1 | 100.0 | 11.2 | 1.7 | 0.2 | 58.3 | 2.6 | 0.4 | 9.1 | 0.5 | 0.3 | 1.1 | 0.1 | 26.1 | 3.2 | 3.2 | 0.4 | 11.2 | 2.9 | 0.0 | 2.5 | 0.0 | 5.4 | 0.0 | 7.8 | 0.8 | 2.8 | * | 7.8 | * |
| Motere Inlet | MT | sheltered urban | 234.9 | 20.1 | 138.5 | 13.8 | 2.5 | 0.1 | 55.0 | 3.0 | 0.2 | 13.7 | 1.2 | 0.2 | 40.9 | 2.3 | 20.8 | 5.4 | 2.2 | 0.5 | 13.3 | 2.9 | 0.0 | 2.2 | 0.0 | 5.1 | 0.1 | 11.0 | 0.9 | 3.0 | * | 8.2 | * |
| Ngamutu Beach | NB | sheltered urban | 167.3 | 8.9 | 86.5 | 4.9 | 1.9 | 0.1 | 46.4 | 3.3 | 0.2 | 10.0 | 0.8 | 0.3 | 42.3 | 2.8 | 15.2 | 0.3 | 1.3 | 0.2 | 6.8 | 3.3 | 0.2 | 2.9 | 0.2 | 6.3 | 0.4 | 7.4 | 0.4 | 3.1 | * | 8.0 | * |
| Otumoetai | OT | sheltered urban | 87.4 | 3.4 | 39.4 | 3.6 | 1.5 | 0.1 | 26.5 | 4.2 | 0.1 | 7.9 | 0.1 | 0.5 | 22.9 | 1.2 | 2.4 | 0.4 | 1.9 | 0.6 | 7.2 | 1.5 | 0.0 | 1.3 | 0.0 | 2.8 | 0.1 | 8.5 | 0.3 | 1.4 | 0.0 | 6.8 | 0.1 |
| Whangamata | WA | sheltered urban | 79.9 | 2.9 | 44.8 | 2.0 | 0.9 | 0.1 | 47.3 | 1.2 | 0.1 | 4.3 | 0.2 | 0.3 | 17.3 | 0.8 | 4.8 | 0.4 | 1.6 | 0.1 | 4.9 | 1.6 | 0.0 | 1.2 | 0.0 | 2.8 | 0.1 | 6.9 | 0.3 | 1.6 | * | 7.8 | * |
| Days Bay | EA | exposed urban | 246.3 | 4.9 | 93.4 | 1.4 | 2.1 | 0.2 | 43.8 | 2.6 | 0.1 | 20.3 | 0.8 | 0.1 | 40.7 | 1.2 | 73.9 | 4.5 | 5.4 | 0.2 | 7.8 | 4.1 | 0.1 | 3.0 | 0.1 | 7.1 | 0.2 | 8.3 | 0.2 | 4.2 | * | 8.8 | * |
| Ferry Terminal | FT | exposed urban | 328.2 | 16.5 | 157.5 | 19.9 | 2.2 | 0.0 | 72.4 | 3.9 | 0.2 | 24.1 | 5.4 | 0.2 | 49.2 | 3.8 | 71.2 | 3.7 | 8.3 | 1.8 | 11.9 | 7.3 | 0.6 | 3.3 | 0.3 | 10.6 | 0.3 | 10.3 | 0.5 | 4.5 | * | 8.4 | * |
| Hardinge Road | HA | exposed urban | 26.4 | 2.7 | 1.1 | 0.0 | 0.4 | 0.0 | 2.7 | 2.2 | 0.4 | 3.5 | 0.5 | 0.6 | 8.3 | 0.7 | 3.2 | 0.2 | 0.1 | 0.1 | 7.4 | 1.1 | 0.0 | 0.9 | 0.1 | 1.9 | 0.1 | 3.4 | 0.3 | 1.1 | 0.1 | 7.7 | 0.0 |
| Moa Point | MO | exposed urban | 179.4 | 8.9 | 69.5 | 5.0 | 1.4 | 0.3 | 50.4 | 4.0 | 0.5 | 12.9 | 2.3 | 0.3 | 57.9 | 1.6 | 21.5 | 0.8 | 4.1 | 0.3 | 8.2 | 7.6 | 0.3 | 3.6 | 0.3 | 11.2 | 0.3 | 6.7 | 0.3 | 3.8 | * | 8.2 | * |
| Point Arther | PA | exposed urban | 273.6 | 32.2 | 110.3 | 8.1 | 2.5 | 0.2 | 44.5 | 2.3 | 0.4 | 20.2 | 2.9 | 0.1 | 43.3 | 2.7 | 81.4 | 16.5 | 3.5 | 1.0 | 10.2 | 3.5 | 0.3 | 2.5 | 0.2 | 6.0 | 0.5 | 9.8 | 1.2 | 3.9 | * | 8.8 | * |
| Tagg Road | TG | exposed urban | 150.5 | 5.3 | 84.4 | 4.3 | 2.1 | 0.1 | 40.7 | 5.7 | 0.4 | 8.3 | 0.4 | 0.7 | 22.3 | 2.7 | 3.9 | 0.9 | 0.6 | 0.1 | 23.3 | 3.2 | 0.0 | 4.0 | 0.1 | 7.2 | 0.1 | 7.0 | 0.2 | 3.0 | * | 10.0 | * |
| Ebbtide Street | ET | enriched urban | 369.8 | 14.6 | 182.0 | 7.6 | 4.0 | 0.2 | 45.3 | 27.0 | 2.4 | 24.5 | 1.3 | 1.1 | 46.6 | 0.4 | 27.9 | 3.8 | 21.0 | 2.4 | 36.7 | 5.4 | 0.0 | 4.6 | 0.3 | 9.9 | 0.3 | 12.8 | 0.5 | 4.0 | 0.0 | 4.8 | 0.0 |
| McCormack's Bay | MC | enriched urban | 205.2 | 5.6 | 94.9 | 3.7 | 1.6 | 0.0 | 58.8 | 6.0 | 0.0 | 11.6 | 0.2 | 0.5 | 26.3 | 0.6 | 40.8 | 1.3 | 15.1 | 0.3 | 8.7 | 4.2 | 0.1 | 3.6 | 0.1 | 7.8 | 0.2 | 8.3 | 0.2 | 3.4 | * | 8.7 | * |
| Onehunga lagoon | ON | enriched urban | 149.2 | 6.0 | 62.6 | 4.6 | 2.5 | 0.0 | 24.7 | 6.8 | 0.6 | 16.9 | 1.5 | 0.4 | 31.4 | 3.1 | 5.2 | 0.3 | 1.7 | 0.1 | 22.1 | 4.6 | 0.1 | 7.1 | 0.2 | 11.7 | 0.3 | 6.8 | 0.3 | 3.1 | 0.0 | 15.2 | 0.0 |
| Saint Kilda discharge | SK | enriched urban | 165.0 | 5.7 | 68.9 | 4.2 | 1.5 | 0.2 | 46.4 | 11.1 | 2.5 | 8.0 | 0.8 | 1.4 | 41.4 | 2.6 | 24.3 | 2.5 | 1.6 | 0.3 | 8.2 | 6.3 | 0.3 | 3.4 | 0.2 | 9.7 | 0.1 | 5.9 | 0.2 | 3.9 | * | 7.4 | * |
| Talleys Factory | TL | enriched urban | 235.5 | 11.1 | 128.9 | 7.0 | 2.3 | 0.1 | 56.7 | 8.5 | 0.1 | 11.9 | 0.6 | 0.7 | 51.6 | 2.6 | 10.8 | 1.5 | 3.5 | 0.1 | 18.1 | 5.8 | 0.2 | 3.7 | 0.1 | 9.5 | 0.3 | 8.2 | 0.4 | 4.0 | * | 7.9 | * |



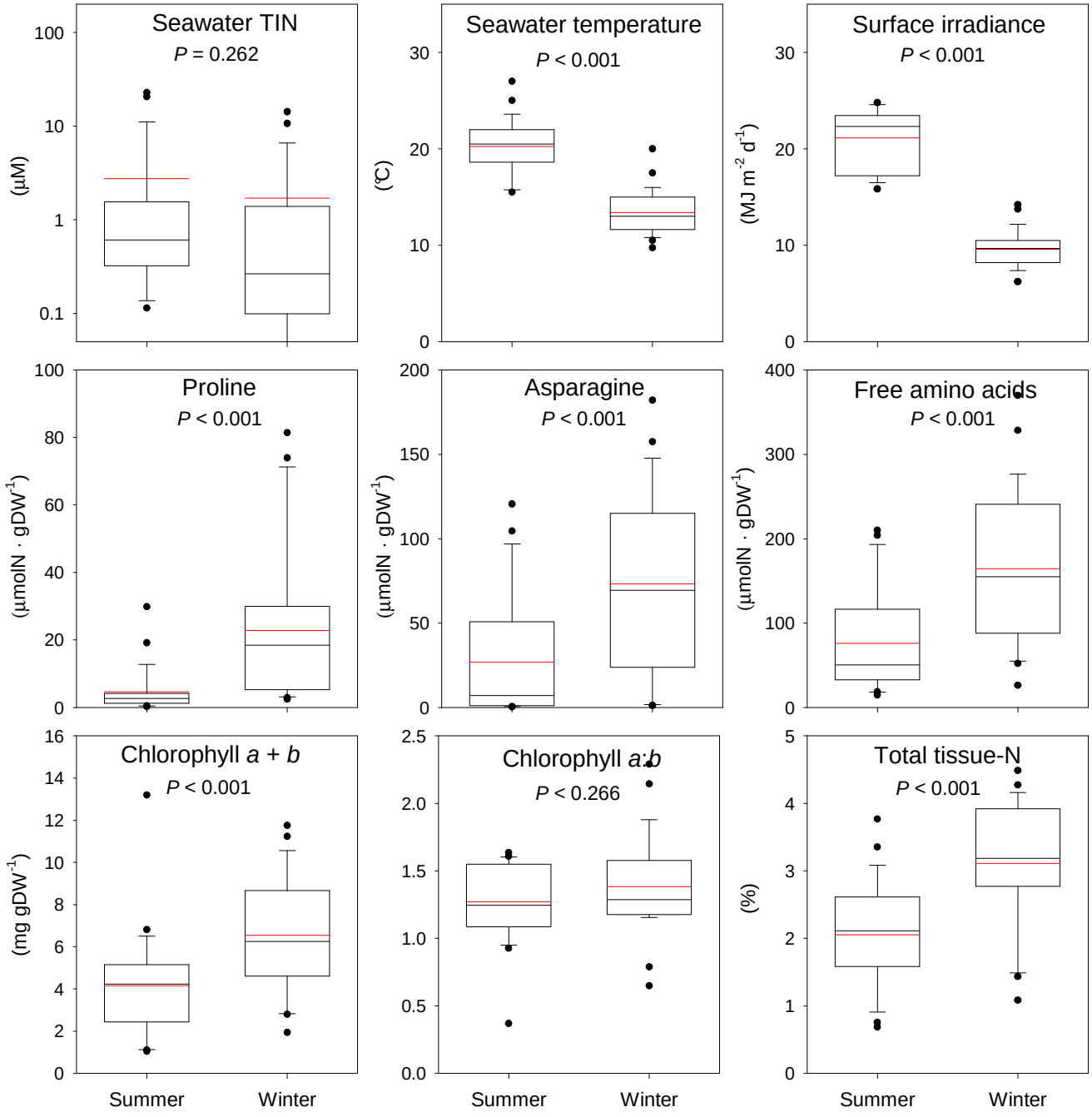

**Appendix 2.8.** Relationship between seawater total inorganic nitrogen and N-indices in *Ulva* for all sites in summer 2002. Values are means ± S.E. for three replicate samples.



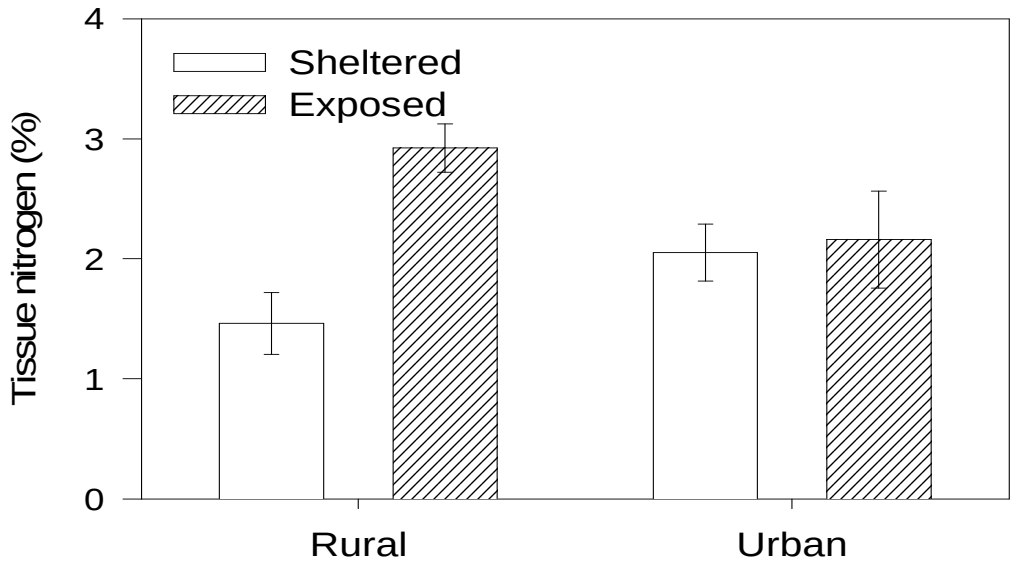

**Appendix 2.9.** Relationship between seawater total inorganic nitrogen and N-indices in *Ulva* for all sites in winter 2002. Values are means ± S.E. for three replicate samples.



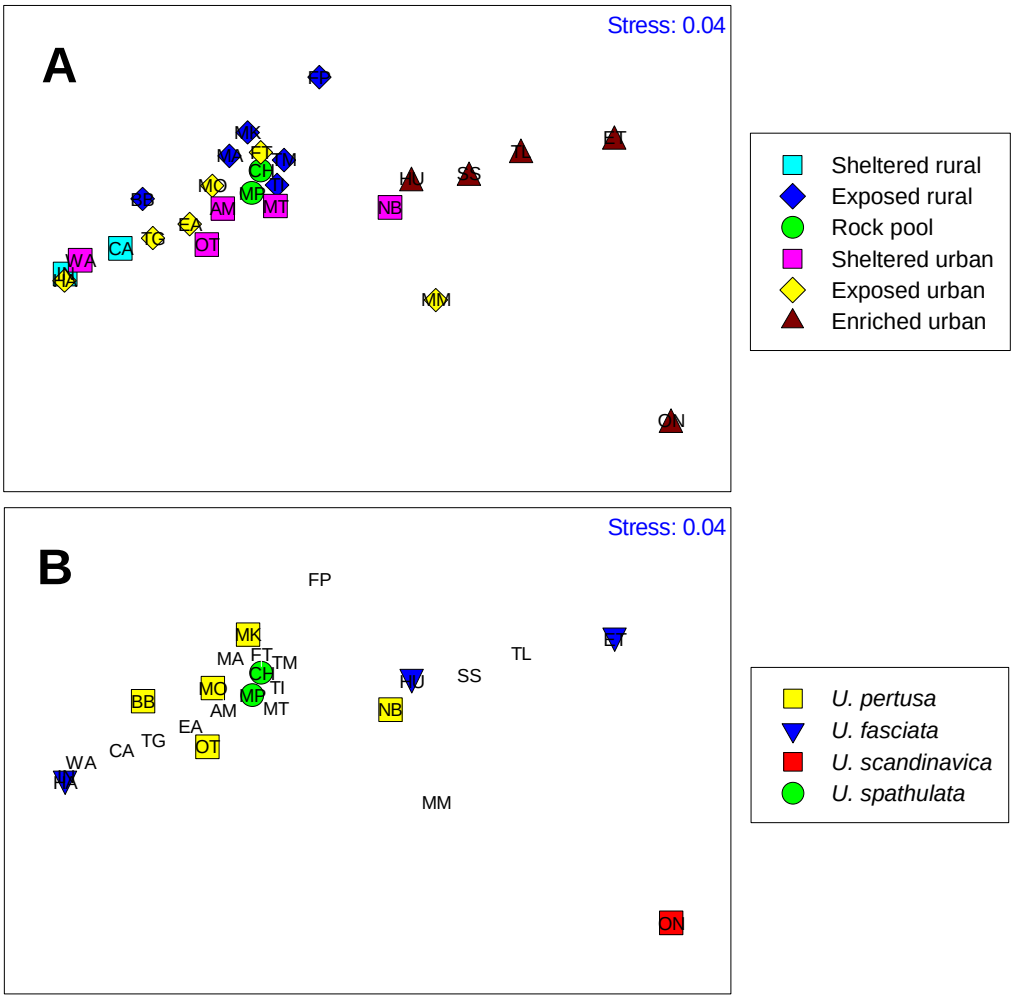

**Appendix 2.10.** Comparison of asparagine content in *Ulva* from different environments in summer (top plot) and winter (bottom plot). Plots are based on the three ordination grouping used in Figures 2.7 and 2.8 (i.e. derived from overall similarity of nitrogen status). Three groups of sites (separated by vertical dashed lines) range from those with (**A**) *Ulva* containing low N-indices, through an intermediate group of sites with (**B**) *Ulva* containing intermediate N-indices, to a group of sites with (**C**) *Ulva* containing high N-indices. Values are means ± S.E. for three replicate samples.



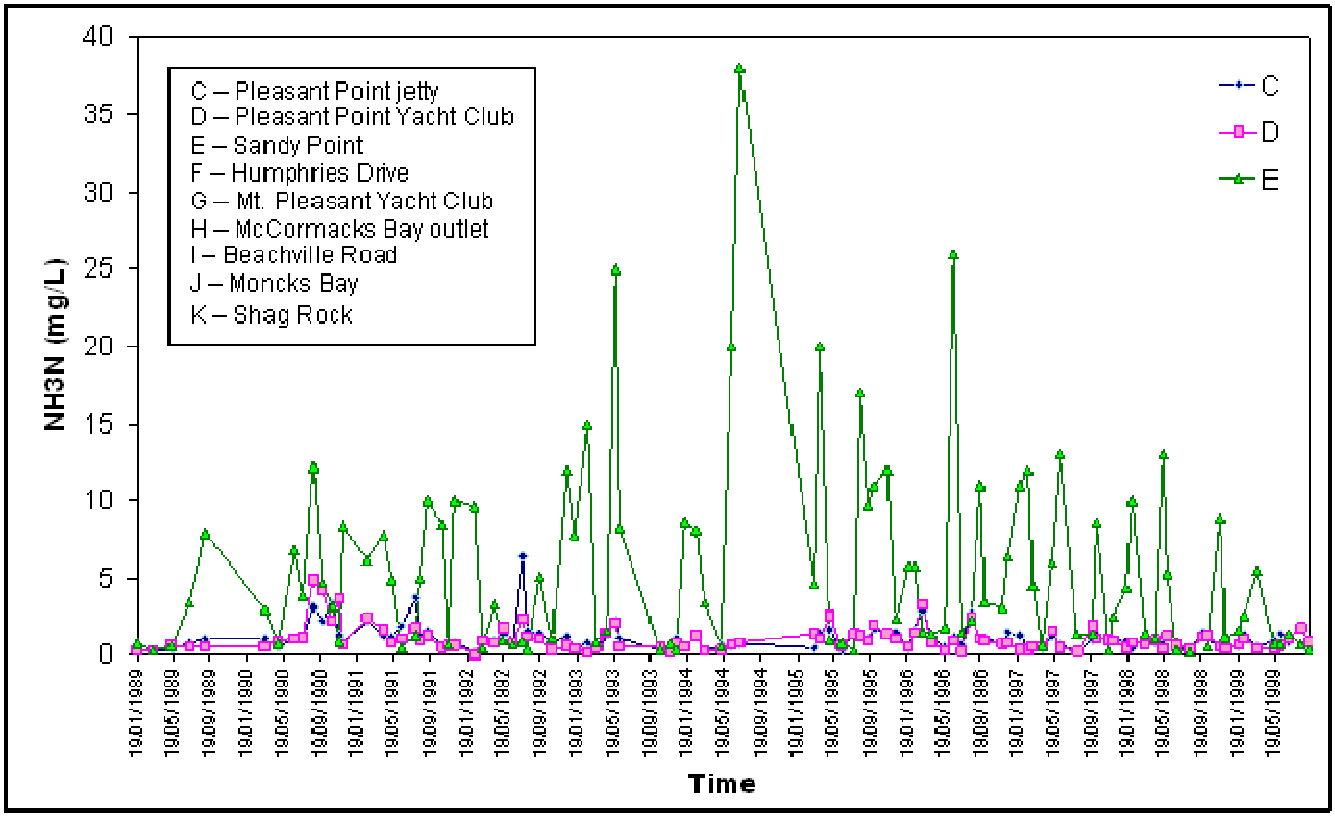

**Appendix 2.11.** Box and whisker plot comparisons (using Mann-Whitney Rank Sum tests) between summer and winter of seawater total inorganic nitrogen (TIN), seawater temperature and surface irradiance, and *Ulva* tissue-N indices of proline, asparagine, total free amino acids, chlorophyll *a* + *b*, chlorophyll *a* : *b* and total tissue-N content. Mean values are indicted as red lines.

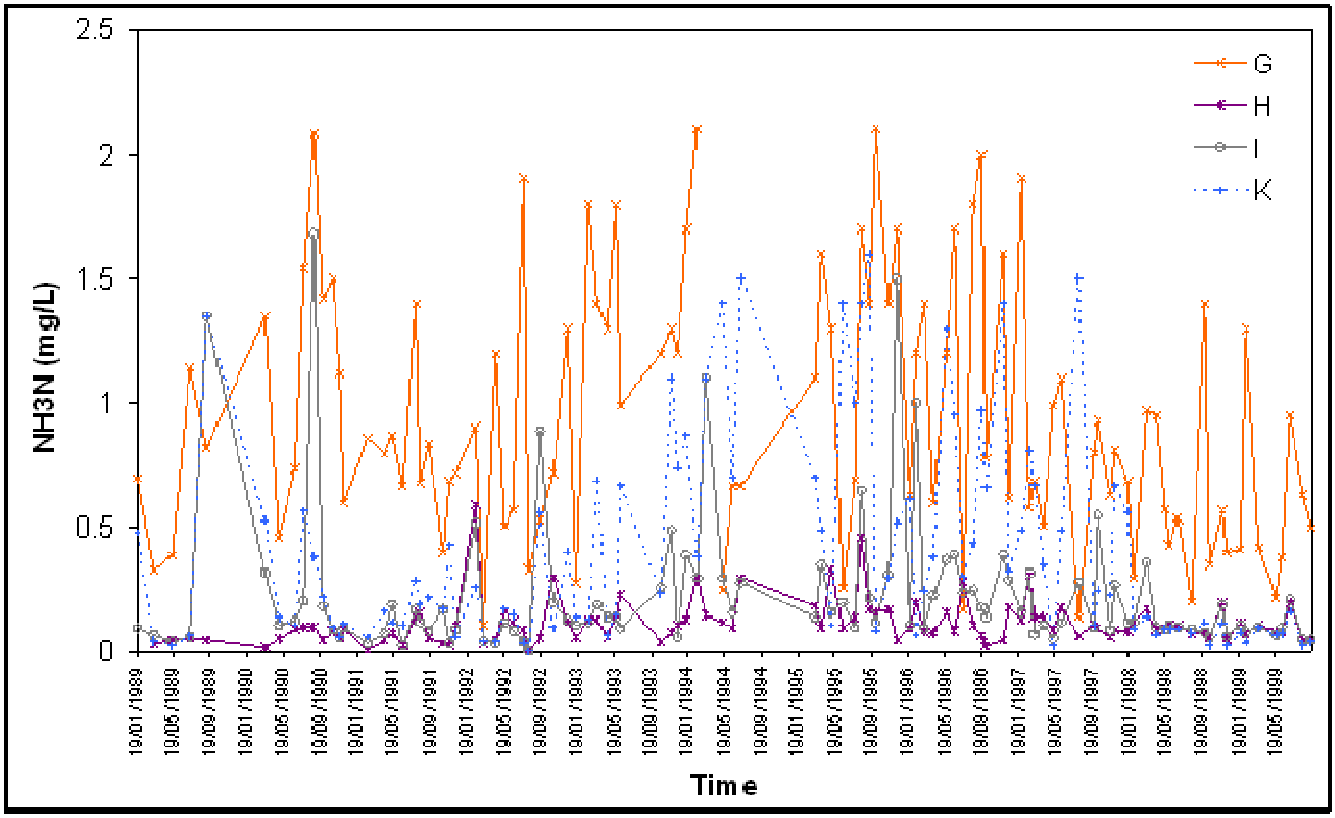



**Appendix 2.12.** Comparison of total tissue nitrogen content in *Ulva* showing interaction of sheltered and exposed sites within rural and urban settings. Note that data are combined for summer and winter, and values are means ± S.E. for three replicate samples.




**Appendix 2.13.** Similarity of nitrogen status in *Ulva* samples (based N-indices of free amino acids, total chlorophyll and tissue nitrogen) from contrasting environments around New Zealand in summer 2003. Symbols in the top plot indicate environmental categories while symbols in the bottom plot indicate *Ulva* species where known.




**Appendix 2.14.** Example of nutrient variability during long-term (10 years) monitoring of ammonium concentrations at sites within the Avon-Heathcote Estuary. Figures kindly provided by Dr. Lesley Bolton-Ritchie, Environment Canterbury.

# Chapter Three
# Experimental assessment of biochemical responses to nitrogen concentration in *Ulva*

## 3.1 Abstract

To determine the relationship between nitrogen concentration in seawater and the responses of biochemical nitrogen indices in *Ulva pertusa*, several experiments were conducted in an outdoor, flow-through culture apparatus. In this apparatus effects of nitrogen concentration, nitrogen source (nitrate and ammonium), light and seawater motion were investigated. In addition, to incorporate effects of contrasting seasons, experiments were conducted in winter, late winter, summer and late summer.

In addition to growth rates in *Ulva*, other biochemical responses to seawater nitrogen availability were measured as N-indices. These indices included tissue nitrogen, chlorophyll, free amino acids and rates of ammonium assimilation. Of these responses, increases in free amino acids, particularly asparagine, provided the strongest indicator of increases in nitrogen availability. Tissue nitrogen and chlorophyll also increased with seawater nitrogen concentration. However, it was apparent that these indices were also strongly influenced by light, and probably season. Rates of ammonium assimilation provided no overall measure of the availability of nitrogen in seawater and were clearly affected by season. Similarly, growth rates in *Ulva* only showed a response to nitrogen addition in summer months.

In addition to the other indices, the stable isotopes of nitrogen ($\delta^{15}N$) in *Ulva* were also examined. As well as providing a clear distinction between natural and synthetic nitrogen sources this parameter also showed only minor fractionation (ranging from 1.3 ‰ to -1.9 ‰) of $^{15}N$ supplied from synthetic nitrate and ammonium under both light-saturating and light-limiting conditions. Therefore it is suggested that in

combination with $\delta^{15}N$, biochemical nitrogen indices in *Ulva* (particularly free amino acids), as quantitative measures of biologically available nitrogen in seawater, are likely to provide useful information about both the amount and composition of nitrogen entering coastal environments.

## 3.2 Introduction

It is widely acknowledged that conventional approaches to measuring seawater nutrient concentrations are limited either because they do not necessarily reflect what is biologically available to primary producers (Lyngby, 1990; Fong *et al.*, 1998) or because they can be highly variable (Björnsäter and Wheeler, 1990; Jones *et al.*, 1996; Valiela *et al.*, 1997; Fong *et al.*, 1998). Moreover, changes in nutrient concentrations alone may be difficult to interpret unless good long-term data is available (Cloern, 2001). In addition, it is often hard to identify the source of potentially harmful excess nutrients in diffuse marine mixing zones. In recognition of these limitations there has been an increasing amount of research in recent years developing macroalgae as indicators of nutrient, particularly nitrogen, availability (Horrocks *et al.*, 1995; Sfriso, 1995; Jones *et al.*, 1996; Fong *et al.*, 1998; McClelland and Valiela, 1998; Costanzo *et al.*, 2000; Barr and Rees, 2003; Cohen and Fong, 2005; Cohen and Fong, 2006).

Most studies of macroalgal indicators of nitrogen loading, such as those above, have focused on responses of biochemical nitrogen indices including total tissue nitrogen, accessory pigments and in some cases amino acids (Jones *et al.*, 1996; Fong *et al.*, 1998; Barr and Rees, 2003; Cohen and Fong, 2005; Cohen and Fong, 2006). However, little is known about changes in free amino acids (both individual and total) in macroalgae in response to changes in nitrogen concentration. In addition to the effects of nitrogen concentration on nitrogen content (and growth) in natural populations of macroalgae it is also known that other factors, including water motion (Lapointe and Ryther, 1979; Parker, 1981; Larned and Atkinson, 1997; Hurd, 2000) and the seasonal effects of light and temperature (Chapman and Craigie, 1977; Rosenberg and Ramus, 1982; Wheeler and Weidner, 1983; Thomas and Harrison, 1985; Duke *et al.*, 1989b; Duke *et al.*, 1989a), are important. For studies that

specifically examine nitrogen composition and source, the stable nitrogen isotopes in macroalgae have been well utilised (McClelland *et al.*, 1997; McClelland and Valiela, 1998; Costanzo *et al.*, 2000; Costanzo *et al.*, 2001; Jones *et al.*, 2001; Gartner *et al.*, 2002; Umezawa *et al.*, 2002; Rogers, 2003; Savage and Elmgren, 2004; Cohen and Fong, 2006).

There are numerous, laboratory-controlled studies that have examined various responses (e.g., nitrogen uptake and assimilation) to nitrogen availability over short (hours to days) time scales (Rosenberg and Ramus, 1984; Harrison *et al.*, 1989; Pedersen, 1994; Rees *et al.*, 1998; Taylor *et al.*, 1998; Campbell, 1999; Taylor and Rees, 1999; Barr *et al.*, 2004; Taylor *et al.*, 2006). However, there are relatively few that have examined longer term biochemical responses to either nitrogen addition (in combination with other factors) (Duke *et al.*, 1989b; Björnsäter and Wheeler, 1990; Coutinho and Zingmark, 1993; Fong *et al.*, 1994; McGlathery *et al.*, 1996) or constant nitrogen concentration (DeBoer *et al.*, 1978; Lapointe and Tenore, 1981; Lapointe and Duke, 1984; Vergara *et al.*, 1993; Andria *et al.*, 1999).

While some responses to nitrogen supply may change quickly (hours, e.g., Barr and Rees (2003)) others, such as total tissue nitrogen content or levels of chlorophyll, may take several days to respond to changes in nitrogen supply (e.g., McGlathery *et al*. (1996)). Moreover, it has been shown that total nitrogen turnover in *Ulva* may take 12 -15 days (Aguiar *et al.*, 2003). Therefore, in order to assess both slow (e.g., tissue nitrogen and chlorophyll content) and rapid (e.g., amino acids) responses to nitrogen loading together it was concluded that the best approach would be to maintain algae at constant concentrations over long periods (days) to achieve equilibrium states.

A simple, reliable and precise seawater flow-through system was designed which was capable of maintaining several (up to 16) different nutrient concentrations over time. The system was constantly supplied with natural, low-nutrient seawater and natural turbulence using waves created by adjustable dump buckets. As well as providing turbulence the dump buckets also mixed the nutrients that were constantly added to growth chambers. Using this apparatus the effects of nitrogen source (e.g., nitrate versus ammonium), nitrogen concentration, light, and seawater turbulence were

investigated. In addition, experiments were conducted in winter, late winter, summer and late summer to incorporate seasonal effects. Given the range of other factors the primary aim of the following experiments was to identify nitrogen indices in *Ulva* that provided the clearest and most reliable indication of nitrogen loading. Growth rates and biochemical responses in *Ulva* including, tissue nitrogen (and $\delta^{15}N$), chlorophyll, free amino acids (and individual amino constituents) and rates of ammonium assimilation, were measured. A total of four experiments were conducted on the green alga *Ulva pertusa* as follows:

Experiment 1. Effect of light and nitrogen addition on N-indices in *Ulva pertusa*. July (winter) 2003.

Experiment 2. Effect of nitrogen concentration on FAA content in *Ulva pertusa*. August / September (late winter) 2003.

Experiment 3. Effect of flow rate and water motion on growth and N-indices in *Ulva pertusa*. March / April (late summer) 2004.

Experiment 4. Effect of light, and ammonium versus nitrate addition on growth and N-indices in *Ulva pertusa*. January (summer) 2005.

## 3.3 Methods

***Algal sampling***

### 3.3.1 Collection of experimental algae

For the following experiments *Ulva pertusa* was collected from Otumoetai, Tauranga Harbour (37° 39' 27.68 S, 176° 8' 31.57 E). Unless otherwise specified, *Ulva* was returned to the Leigh Laboratory where it was kept under natural light in running seawater supplied continuously from the Leigh Laboratory's seawater system (using natural Goat Island seawater) for at least one week prior to the commencement of any experiment to allow tissue to equilibrate to local conditions. Generally *Ulva* tissue was maintained in good condition while under culture, however on a few occasions sporulation occurred in *Ulva* tissue (particularly in non-enriched controls). On these occasions sporulated tissue was removed, and therefore excluded from growth and biochemical analysis.

Biochemical responses may show diel changes (e.g., some amino acids and their associated enzymes). Consequently in the following outdoor experiments (and sampling in the following chapters) sampling was always conducted close to the middle of the day for purposes of standardisation.

### 3.3.2 Sub-sampling *Ulva* thalli

*Ulva* thalli were sub-sampled as described in Chapter 2.

***Analytical***

### 3.3.3 Free amino acids

Amino acid samples were extracted from 1 g fresh weight of *Ulva* tissue as described in Chapter Two with the following modifications. All extractions were done on ice in a polystyrene cooler bin. Tissue samples were placed into a 20 ml scintillation vial to which 5 ml 1M perchloric acid was quickly added. The vial was then capped, shaken and left on ice for 10 min before neutralising with 5 ml 1M KOH/0.2 M MOPS. After 60 minutes on ice 1 ml supernatant was drawn off (while avoiding any perchlorate precipitate), using a fresh plastic eyedropper pipette, and placed into labelled 1.5 ml microcentrifuge tubes. These were stored at - 80 °C for later HPLC analysis of amino acid composition as described by Barr and Rees (2003) (See also Chapter Two). After rinsing with distilled water extracted tissue was dried to a constant weight at 65 °C.

Unknown amino acids (i.e., U2 $^{9.48}$) and co-eluting amino acids (i.e., glycine and citrulline) were treated as in Chapter Two. Values for all amino acids (and their ratios) are expressed in μmol N · g $DW^{-1}$.

### 3.3.4 Chlorophyll

Chlorophyll was extracted and determined as described in Chapter Two. Values are expressed as mg · g $DW^{-1}$.

### 3.3.5 Tissue nitrogen

Tissue nitrogen (in %) and $\delta^{15}N$ (in ‰) content was determined as described in Chapter Two.

### 3.3.6 Growth

Growth rate in *Ulva* was measured as the change in fresh weight biomass over time. Prior to weighing, excess surface water was removed by placing thalli in a Zeiss salad spinner and spinning a few times. This operation, which typically took less than minute to perform, was done as quickly as possible to minimise any affects of handling. Growth was expressed as daily specific growth rates, calculated as:

$$\text{Growth} = \ln \frac{\text{final mass} / \text{initial mass}}{\text{time interval (day)}}$$

### 3.3.7 Determination of maximum rate of ammonium assimilation

Rates of ammonium assimilation were determined using the method described by Rees *et al* (1998) and modified as described by Barr and Rees (2003). Further modification to the method of Barr and Rees (2003) was the use of 1 g fresh weight portions of homogenised (as described in Chapter Two) *Ulva* tissue incubated in 400 μM ammonium in 250 ml seawater for 60 minutes.

### 3.3.8 Seawater sampling and nutrient analyses

Seawater samples were generally taken at the same time as algal tissue samples. Samples were kept chilled on ice and analysed for ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$) and phosphate ($PO_4^{3-}$) with reference to standard curves containing known concentrations of nutrients. Ammonium was determined as described by Koroleff (1983a) and nitrite by Parsons *et al* (1984). Nitrate was reduced to nitrite by passing 20 ml of seawater sample through a cadmium column, and then determined using the method described by Parsons *et al* (1984). Phosphate was determined as described by Koroleff (1983b). Total inorganic nitrogen (TIN) concentrations were calculated as the sum of $NH_4^+$, $NO_2^-$ and $NO_3^-$. The ratio of nitrogen : phosphorus (N : P) was calculated in molar ratios as TIN : $PO_4^{3-}$. All concentrations values are expressed in μM.

### 3.3.9 Statistical analysis

Regression lines were fitted by ordinary least squares in SigmaStat 3.1. Means were compared using a two-way or three-way general linear model (analyses of variance).

The Holm-Sidak method was used to compare among means. Where assumptions of normality or equal variance were not met by the data then comparisons were made using Kruskal-Wallis analysis of variance on ranks.

***Laboratory Apparatus***

### 3.3.10 Seaweed enrichment system

An outdoor (i.e., under natural light) seaweed on-growing apparatus was constructed for maintaining and manipulating *Ulva* under different turbulent, light and nutrient regimes. Sixteen individual seaweed growth chambers were constructed from commercial-grade polypropylene containers measuring 0.47 m long by 0.18 m wide and 0.15 m deep. Each container had a liquid volume of 4.2 L, which was determined by the height of an elongated, drain weir cut in one end (Figure 3.1). A turbulent,

**Plate 3.1.** Outdoor seaweed culturing system comprised of 16 plastic bins housed in a polypropylene drainage bund.

seawater-flow design was incorporated into the growth chambers by using 'dump buckets' constructed from sections of PVC guttering. These were pivoted on 6 mm PVC shafts through one end of each growth chamber. Each dump bucket had an

adjustable nylon set screw which was used to alter the balance point at which a given seawater volume was tipped from the bucket into the growth chamber (Figure 3.1). Tipping volumes were adjustable between 0.1 and 1.2 L. Seawater was supplied continuously to the experimental apparatus from the Leigh Marine Laboratory's seawater system using natural Goat Island seawater. Incoming seawater was coarsely filtered (< 200 μM) to remove any large objects that could cause blockages in the apparatus. A constant head of seawater was maintained using a small (20 L) polypropylene header tank situated 1.2 m above the growth chambers. The seawater head was maintained in the header tank using two, 20 mm agricultural ball-cock valves.

The sixteen growth chambers were arranged in two rows of 8 growth chambers housed in a large (2.4 × 1.2 m) polypropylene drainage bund (Plate 3.1). Each row of eight chambers was supplied with seawater directly from the header tank via a supply manifold of sufficient internal diameter (32 mm) to ensure that frictional losses (and therefore any pressure gradients along the length of the manifold) were minimised. In addition, the manifolds were ring fed (supplied at both ends) to further minimise pressure gradients. From the manifold, water was supplied to individual growth chambers (via the dump bucket) through a flow-regulating nozzle constructed from short lengths (40 mm) of plastic tubing. Depending on the desired seawater flow rate required for a particular experiment, nozzles were selected depending on their internal diameter. In most cases nozzles were made from polyethylene irrigation tubing with a 3.5 mm internal diameter which supplied constant flow rates of close to 1.2 $L \cdot min^{-1}$. When a lower range of flow rates (between 0.1 and 1.0 $L \cdot min^{-1}$) was required, nozzles were fashioned from 200 μL tapered plastic pipette tips cut to different lengths providing a range of nozzle diameters. In other cases when flow rates higher than 1.2 $L \cdot min^{-1}$ were required, lengths of PVC tube (of up to 10 mm internal diameter) were used for flow rates of up to 7 $L \cdot min^{-1}$. For experiments that specifically examine the effect of seawater flow rate and turbulence, values are normalised to average horizontal bulk flow velocities through individual culture chambers in units of $cm \cdot min^{-1}$.

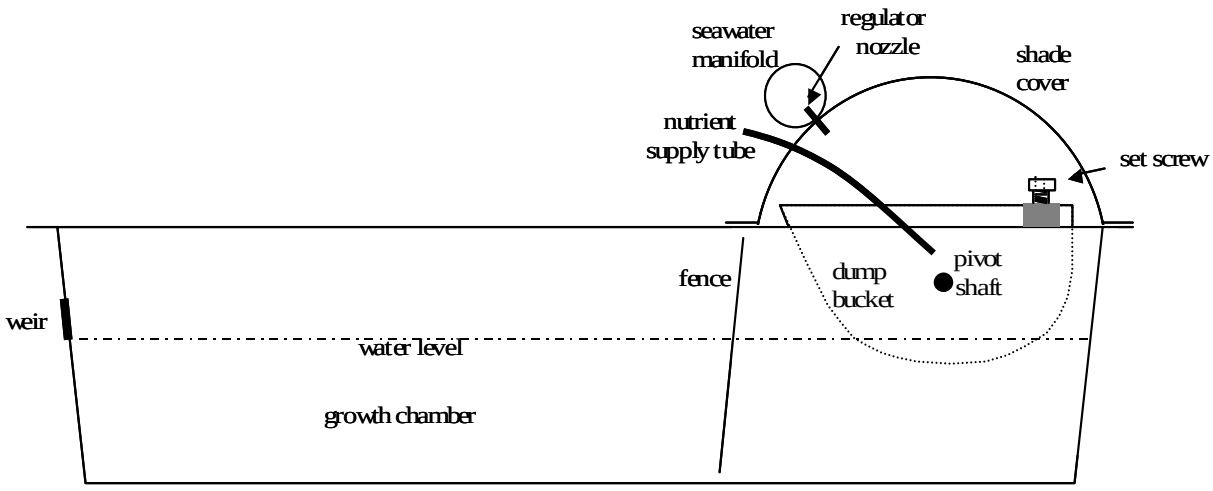



**Figure 3.1.** Schematic plan of individual growth chamber incorporating dump bucket and nutrient supply tube controlled by a peristaltic pump.

Either concentrated nutrients or distilled water only (for the control treatments) were continuously added into the dump buckets from stock solutions containing various combinations (as required) of ammonium chloride ($NH_4Cl$), sodium nitrate ($NaNO_3$) and / or dihydrogen-phosphate ($NaH_2PO_4$) with delivery flow rate controlled by a multi-channel peristaltic pump (supplied with long-life Tygon™ tubes). Note that ortho-phosphate was initially used as a phosphate source until it was found that it formed a precipitate (presumably resulting from a reaction with the concentrated ammonium chloride), which on two occasions blocked the narrow-diameter, nutrient-feed tubes (see Figure 3.2 above). This problem did not occur when dihydrogen-phosphate was adopted as the phosphate source. Nutrients were continuously mixed with seawater by both the effect of entrainment of the seawater jet (from the flow regulating nozzles) onto the end of the nutrient tube and also because of the dumping action when the bucket tipped. Initial experiments showed that the only concentration gradient that was detected in the culture chambers was due to the presence of the alga itself (i.e., uptake resulted in small but measurable reductions in nutrient concentrations between the input dump end and the drain end).

The final concentration of stock solution was adjusted to suit each chamber depending on its measured seawater flow rate. The variation in seawater delivery

flow rates (for the most commonly used rate of 1.2 L · $min^{-1}$) across all 16 chambers was typically less than 3 %. Nutrient concentrations in culture chambers were regularly monitored, and either stock concentrations or peristaltic pump flow rate adjusted as necessary. In addition, ambient (Goat Island) seawater nutrient concentrations were independently monitored on a weekly basis (Dobson, unpublished data) and were factored into all concentration estimations. For example, while controls typically had no nutrients added their concentrations were specified as: those measured (relative to seawater blanks taken from the seawater supply) in each chamber plus ambient seawater nutrient concentration independently measured at the time of the experiment.

In most cases where nitrogen was added (either as nitrate or ammonium) it was at 10 μM because preliminary experiments suggested that biochemical responses began to saturate at concentrations higher than this. Also, since concentrations in the order of 10 μM were commonly measured around New Zealand (see Chapter Two) this value is ecologically realistic. A nitrogen : phosphorus ratio of 10 : 1 was chosen to ensure that no phosphorus limitation occurred in algae that were also enriched with nitrogen. Macroalgal growth becomes phosphorus limited with tissue N : P values above 30 : 1 (Atkinson and Smith, 1983).

***Experiments***

All experiments in this chapter were carried out in the apparatus described above and are presented in chronological order.

**Experiment 1. Effect of light and nitrogen addition on N-indices in *Ulva pertusa*. July (winter) 2003.**

The effect of light and nitrogen addition on N-indices in *U. pertusa* was examined in an orthogonal (two-way) experiment. The entire experiment was run over a period of five weeks from 24 June to 31 July, 2003. Light treatments consisted of either ambient light (light-saturated) or shaded (light-limited) using three layers of neutral density shade cloth. Measurements taken at midday showed that shaded chambers had 18 % of the photosynthetically active radiation (PAR) compared with ambient light chambers. Nitrogen treatments consisted of natural Goat Island seawater (supplied at a rate of 1.2 L · $min^{-1}$) with either no added nutrients or ammonium

supplied at a constant concentration of 10 µM (with phosphorus added to give an N : P = 10 : 1). Seawater nutrient concentrations were monitored on a weekly basis for the duration of the experiment. During the last week of the experiment, two nutrient feed tubes blocked and as a result *Ulva* in these chambers was not used in the analysis of the final week's data (see Figure 3.2 below).

Four *U. pertusa* thalli each weighing approximately 3 g were assigned to each of the sixteen culture chambers in a randomised design. *Ulva* was maintained under ambient light and no added nutrients for one week prior to the start of the experiment to equilibrate algae to these conditions. At the end of the first week, shade screens and / or nutrient additions were applied to the respective treatments for the remainder of the experiment. At weekly intervals, total *Ulva* growth in each chamber was recorded. After weighing, one individual from each chamber was sampled and analysed for amino acid and chlorophyll content as described above. The remaining thalli were then returned to their respective culture chambers for the next weekly growth period. This procedure took approximately 5 minutes per chamber.

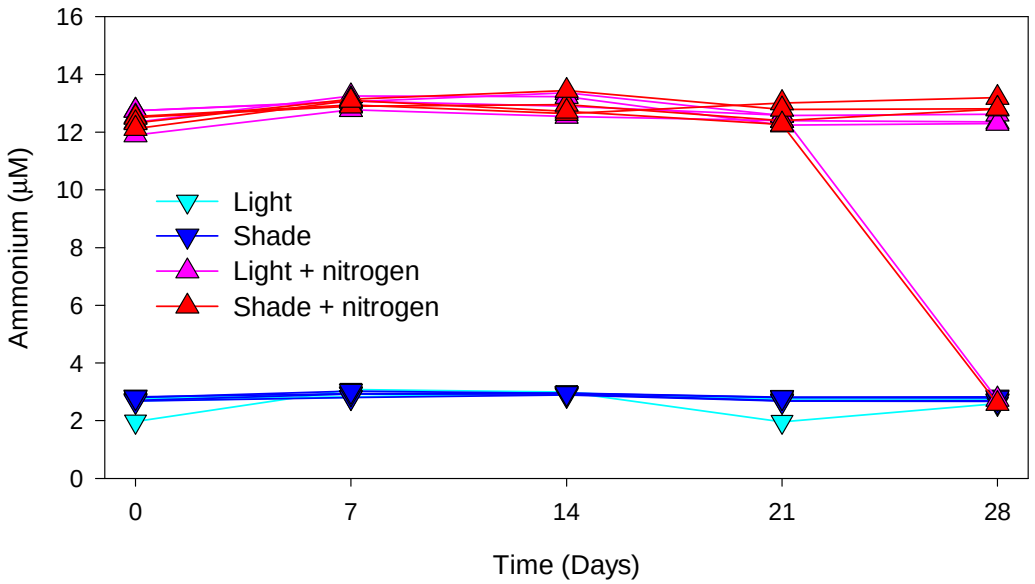



**Figure 3.2.** Ammonium concentrations in 16 culture chambers over duration of Experiment 1. Note that two nutrient feed tubes for ammonium-enrichment treatments had blocked during the last week and therefore results for these two chambers were dropped from analysis for the last time point.

Over the duration of the experiment control seawater TIN concentrations ranged from 2.0 to 3.2 μM with a mean concentration of 2.6 ± 0.2 μM. Solar radiation and seawater temperature averaged 7.3 MJ · $m^{-2}$ · $d^{-1}$ and 15.0 °C (Evans, unpublished data) respectively, over the course of the experiment. At the end of the experiment both tissue nitrogen content and the rates of ammonium assimilation in *Ulva* were measured as described above.

**Experiment 2. Effect of nitrogen concentration on FAA content in *Ulva pertusa*. August / September (late winter) 2003.**

This experiment was conducted to examine changes in nitrogen and FAA content in *Ulva* in response to nitrogen concentration. This experiment was done as two parts, Experiment 2a and 2b. The first examined the effect of high (10, 20 and 40 μM) nitrogen concentrations on *Ulva* N-indices and ran from the 7 to 15 August, 2003. The second examined the effect of low (2, 4, 6, 8, 10, 15 and 20 μM) nitrogen concentrations and ran from the 23 August to the 9 September 2003. Prior to the start of each experiment, *Ulva* was equilibrated to low nitrogen conditions by placing small (≈ 3 g) thalli in plastic chambers under fluorescent lights (at a photon flux density of 350 μE · $m^{-2}$ · $s^{-1}$ [photosynthetically active radiation with a 12 : 12 h light/dark cycle] using Phillips New Generation TLD 36W / 86500$^{o}$ Kelvin colour temperature) for one week. Goat Island seawater was automatically added (controlled by a time-clock and 24 Vac solenoid valve) via an 80 μm filter for 1 minute every hour. An average daily seawater flow rate was chosen such that the water volume (4 L) of each growth chamber was replaced approximately once per day. Turbulence was supplied to the chambers using perspex paddles connected by an eccentric cam to a shaft, which was in turn driven by a wind-screen wiper motor (see Figure 4.2 and Plate 4.2 in Chapter Four).

After one week in the apparatus described above, individual *Ulva* thalli were then added to each of 16 culture chambers under natural light and seawater (constantly supplied at a rate 1.2 L · $min^{-1}$) containing either no added nutrients (control) or constant concentrations of ammonium (10, 20 and 40 μM) plus phosphate at N : P = 10 : 1. Controls and ammonium treatments were represented by four separate replicates each. *Ulva* was maintained under these conditions for 9 to 10 days (from

Experiment 1 it was shown 7 days was the minimum period it took for FAA content to respond to seawater concentration), then sampled and analysed for biochemical content as described above. The whole procedure was repeated again for the second part of the experiment (Experiment 2b) with freshly collected *Ulva*. However, ammonium concentrations were altered to 2, 4, 6, 8, 10, 15 and 20 μM (plus phosphate at N : P = 10 : 1). Since there were only sixteen chambers replicates were repeated over a period of three weeks. Fresh weight growth in *Ulva* was recorded during the last 5 days of each experiment.

Over the duration of both experiments control seawater TIN concentrations ranged from 1.2 to 1.7 μM with a mean concentration of 1.5 ± 0.3 μM. Solar radiation and seawater temperature averaged 10.9 MJ · $m^{-2}$ · $d^{-1}$ and 14.1 °C, respectively (Evans, unpublished data), over the course of the experiment.

**Experiment 3. Effect of flow rate and water motion on growth and N-indices in *Ulva pertusa*. March / April (late summer) 2004.**

The effect of water motion on nitrogen indices and growth rate in *U. pertusa* was examined in three repeated orthogonal experiments from 8 March to 2 April 2004. Three separate growth experiments, each run for seven days, were conducted from the 8 March to 2 April 2004. Sixteen growth chambers were set up for the experiment, eight chambers for turbulent flow treatments and eight for non-turbulent flow treatments. Eight different seawater flow rates ranging from 0.1 to 7.0 L · $min^{-1}$ were set up for each of the turbulent and non-turbulent flow regimes using a combination of different nozzles sizes. These flow rates translated into average horizontal flow velocities through each chamber ranging from approximately 1 cm · $min^{-1}$ to 60 cm · $min^{-1}$. For all turbulent flow treatments buckets were calibrated to tip at a volume of 0.75 L and for non-turbulent flow treatments buckets were fixed in a tilted-down position (by locking the bucket pivots with foam inserts) such that the seawater inflow from the nozzles was entrained directly into the growth chambers. Un-enriched Goat Island seawater was continuously supplied to the apparatus for the duration of the experiment.

In preparation for each experiment, small (≈ 3 g) individual *U. pertusa* thalli were weighed and were randomly assigned to each of the sixteen growth chambers. A small transparent 'fence' constructed of six 3 mm polycarbonate rods was placed above the *Ulva* to ensure plants were constantly submerged under low flow conditions. This modification was added to long-term experiments after observing that there was a tendency for *Ulva* thalli to float and bunch at the drain end of the culture chambers (i.e., the plastic rods ensured that thalli were constantly immersed and spread out in the chambers). One day was allowed for *Ulva* to acclimate to the chambers and time zero fresh weights recorded as described above. Fresh weights were obtained every day until the completion of the trial at day seven. At the end of each trial *Ulva* tissue was analysed for biochemical content as described above. The apparatus was then cleaned, set up again and the entire procedure repeated for the two remaining replicate trials.

Over the duration of the experiment the general apparatus and nozzles were checked regularly. Control seawater TIN concentrations ranged from 2.1 to 3.2 μM with a mean concentration of 2.5 ± 0.4 μM. Solar radiation and seawater temperature averaged 16.8 MJ · $m^{-2}$ · $d^{-1}$ and 18.7 °C, respectively (Evans, unpublished data), over the course of the experiment. Growth was measured every two days and FAA were extracted and analysed at the end of the experiment as described above.

**Experiment 4. Effect of light, and ammonium versus nitrate addition on growth and N-indices in *Ulva pertusa*. January (summer) 2005.**

The effect of light and nitrogen source (nitrate versus ammonium) on nitrogen indices and growth in *U. pertusa* was examined in an orthogonal experiment run over two weeks from 8 January to 22 January 2005. This experiment was done jointly with Bruce Dudley from Victoria University. Light treatments consisted of either ambient light or shade using three layers of neutral density shade cloth as described for Experiment 1. Shaded treatment chambers had 18 % of the PAR compared with ambient light treatments. Nitrogen treatments consisted of natural seawater (supplied at a rate of 1.2 L · $min^{-1}$) with either nitrate or ammonium supplied at a constant concentration of 10 μM (plus phosphate at N : P = 10 : 1).

Small (≈ 3 g) *U. pertusa* thalli were weighed and randomly assigned to each of the culture chambers. Each of the four light and nitrogen treatments was replicated three times in 12 chambers (i.e., 3 × light + ammonium, 3 × light + nitrate, 3 × shade + ammonium, 3 × shade + nitrate) in a randomised block design. Since there were only 16 chambers in total and four chambers remaining for the un-enriched, light-saturated and light-limited reference treatments it was necessary to represent these with one replicate thallus in one chamber and two pseudo-replicated thalli in the other (i.e., two chambers were used for each treatment). However, since the main purpose of this experiment was to compare the difference between nitrogen source (under the two contrasting light regimes) the semi pseudo-replication of un-enriched treatments was not considered important and values obtained from them were only used for general reference to un-enriched conditions. They were not included in any statistical comparisons.

Over the course of the experiment ambient seawater nitrogen concentration ranged from 1.5 to 2.0 μM with a mean concentration of 1.8 ± 0.1 μM. Solar radiation and seawater temperature averaged 27.2 MJ · $m^{-2}$ · $d^{-1}$ and 18.4 °C, respectively (Evans, unpublished data), over the course of the experiment. Growth was measured every two days and FAA were extracted and analysed at the end of the experiment as described above.

## 3.4 Results

**Experiment 1. Effect of light and nitrogen addition on N-indices in *Ulva pertusa*. July (winter) 2003.**

From the start of the experiment growth rates in *Ulva* under ambient light were considerably higher than those in shade, irrespective of nitrogen addition (Figure 3.3). There was an overall increase in growth rate in *Ulva* for both ambient light treatments (i.e., for *Ulva* with and without added nitrogen) compared with shaded treatments, but growth was variable over the whole period. Growth rates in the shaded treatments showed a very similar variation through time, but remained low throughout the experiment with only a minor increase by day 28 (Figure 3.3). At the end of the experiment the highest growth rate recorded in the experiment (0.101 ± 0.004 $day^{-1}$)

was in light, non-enriched *Ulva*. Although overall growth rates were higher in light treated *Ulva*, there was an obvious similarity in the variation of growth rates over time between light and shaded treatments. However, this variation did not correlate with any external factor (i.e. changes in light, temperature or ambient nutrient concentration). At the end of the experiment there was no significant effect of nitrogen addition on growth rate in *Ulva* according to comparison made by two-way ANOVA (Table 3.1).

Chlorophyll content in *Ulva* showed a general increase for all treatments throughout the experiment with respect both to initial native values and time-0 values (Figure 3.4). However, by the end of the experiment levels of chlorophyll *a* and *b* in *Ulva* were the lowest in the light treatment that had no nitrogen added. Chlorophyll *a* levels were similar in shade, shade plus nitrogen and light plus nitrogen treatments by day 28 (Figure 3.4, A). The increase in chlorophyll *a* over the duration of the experiment had slowed by day 28 for these three treatments and had declined for the light treatment relative to day 21. Conversely, chlorophyll *b* levels in the shaded treatments (both with and without added nitrogen) increased throughout the experiment while chlorophyll *b* in both the light treatments had reached a plateau in values in the last seven days (Figure 3.4, B). This observation suggested that chlorophyll *b* was slower to respond (presumably as a photoacclimation response) in lower light conditions. Continuing increases in total chlorophyll content (TChl, chlorophyll *a* + *b*) throughout the experiment were also apparent in the shaded treatments while the nitrogen-enriched, unshaded *Ulva* had reached a plateau in the last week of sampling (Figure 3.4, C).

Amino acids showed very different trends compared to those of chlorophyll. The total free amino acid (FAA) pool and asparagine showed elevated levels in nitrogen-enriched *Ulva* (light and shade treatments) compared with their respective controls (Figure 3.5). Relative to asparagine, glutamine levels were both low (ranging from 1.2 to 4.4 μmol N · g $DW^{-1}$) and variable throughout the experiment. However, as with asparagine, nitrogen-enriched *Ulva* (relative to their respective controls) had higher levels of glutamine (Figure 3.5). In the last week of the experiment there was a marked decline in glutamine levels (relative to total FAA), which may have related to the higher growth rate in light treatments during the same period.

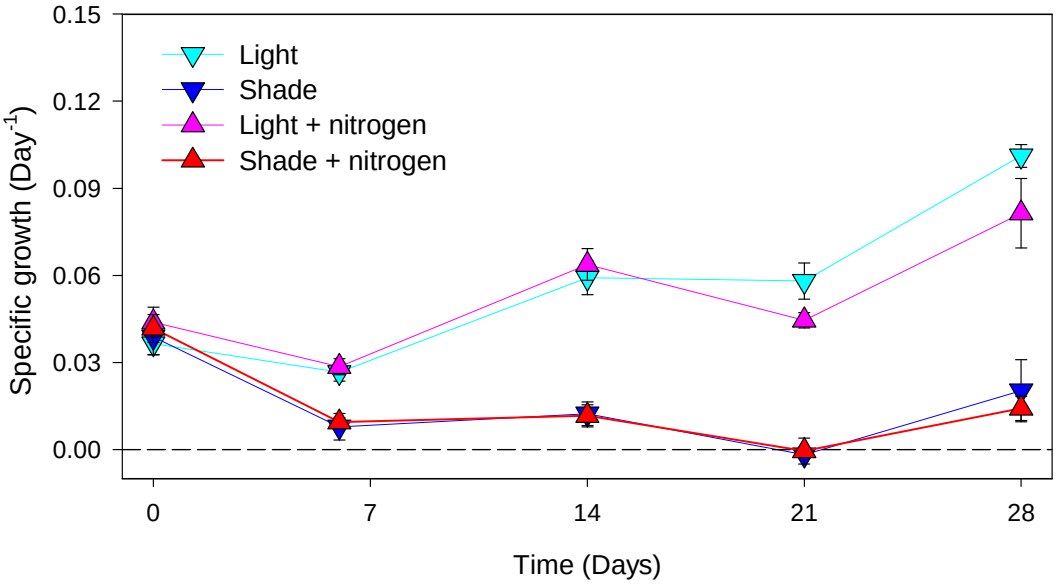



**Figure 3.3.** Changes in growth rate in *Ulva pertusa* in culture chambers maintained under either ambient light or shade, with and without nitrogen addition. Values are means ± standard errors for four separate replicates.

**Table 3.1.** Two-way analysis of variance of growth rate (day 21 – 28) for *Ulva pertusa* maintained under either ambient or shaded light, with and without nitrogen addition.

**Two-way ANOVA on differences in growth rate in *Ulva***

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 0.0188 | 0.0188 | 66.27 | <0.001 |
| Nitrogen | 1 | 0.0006 | 0.0006 | 2.03 | 0.185 |
| Light × Nitrogen | 1 | 0.0002 | 0.0002 | 0.57 | 0.469 |
| Residual | 10 | 0.0028 | 0.0003 | | |
| Total | 13 | 0.0233 | 0.0018 | | |

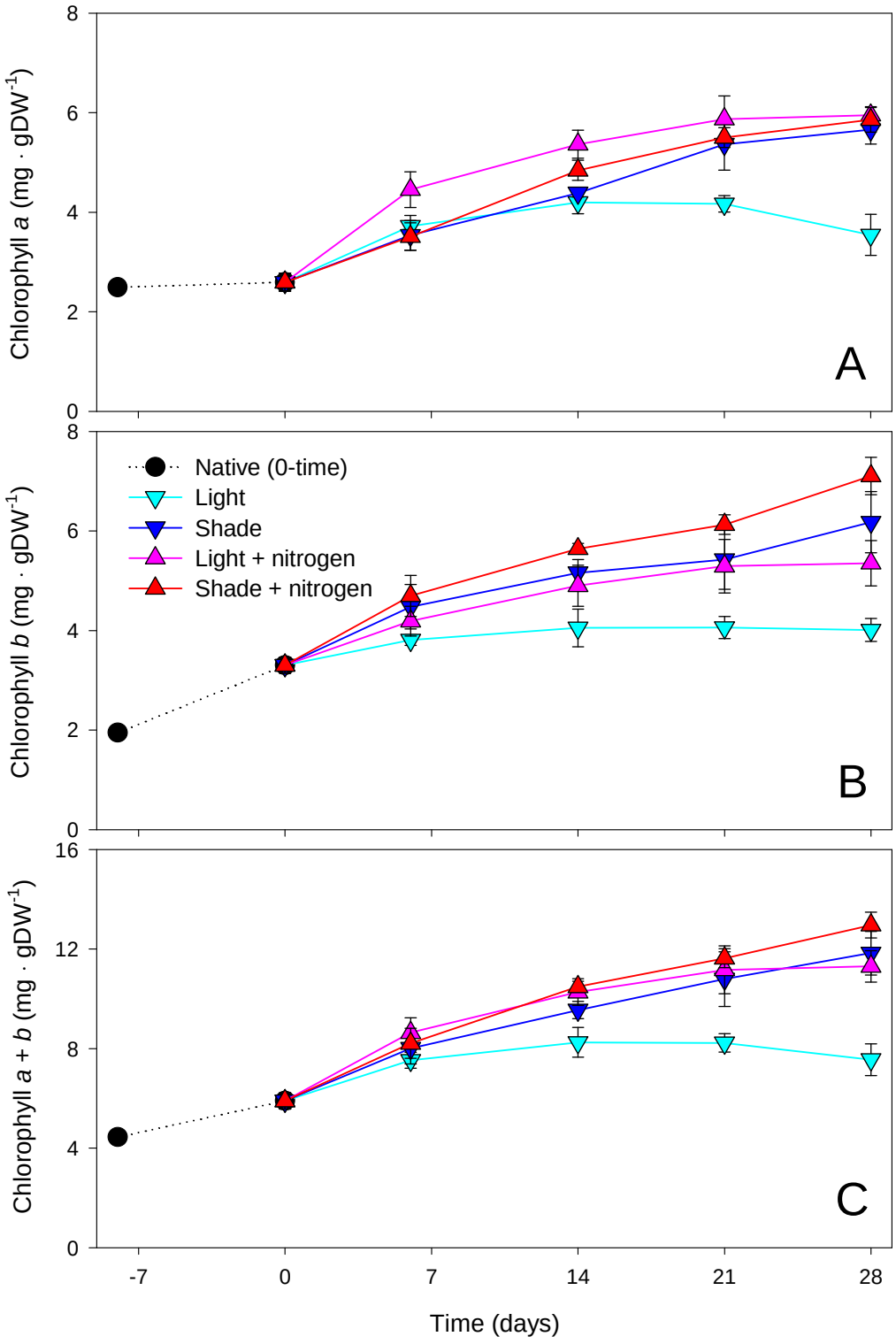



**Figure 3.4.** Changes in (**A**) chlorophyll *a*, (**B**) chlorophyll *b* and (**C**) total chlorophyll content in *Ulva pertusa* in culture chambers maintained under either ambient or shaded light, with and without nitrogen addition. Values are means ± standard errors for four separate replicates.

At the end of the experiment (at day 28) there was a clear visual difference in thalli greenness and chloroplast content in *Ulva* treated with light alone clearly being paler than the other three treatments (Plate 3.2). There was a significant effect of both light treatment and nitrogen addition on TN, TChl and FAA content in *Ulva* (Figure 3.6). In all cases, unshaded controls had the lowest values of TN, TChl and FAA content. However, TN and TChl content in the shaded controls were not significantly different from their unenriched counterparts (Figure 3.6, A and B). In addition, although FAA content in *Ulva* from the shaded control was higher than the unshaded control, after 28 days this N-index was less affected by lower levels of light than either TChl or TN content (Figure 3.6, C).

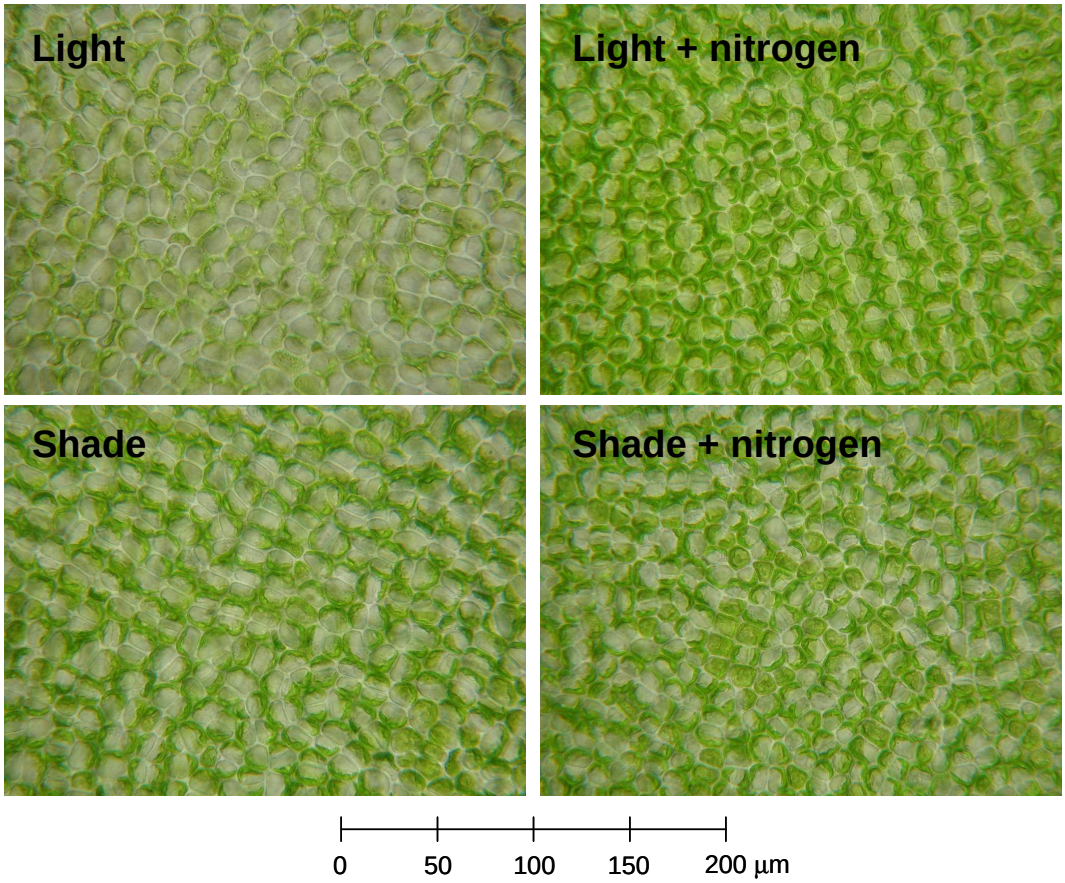



**Plate 3.2.** Comparison of chloroplasts in *Ulva pertusa* maintained under either ambient or shaded light, with and without nitrogen addition. The Individual plates shown above are representative examples of the four treatments.

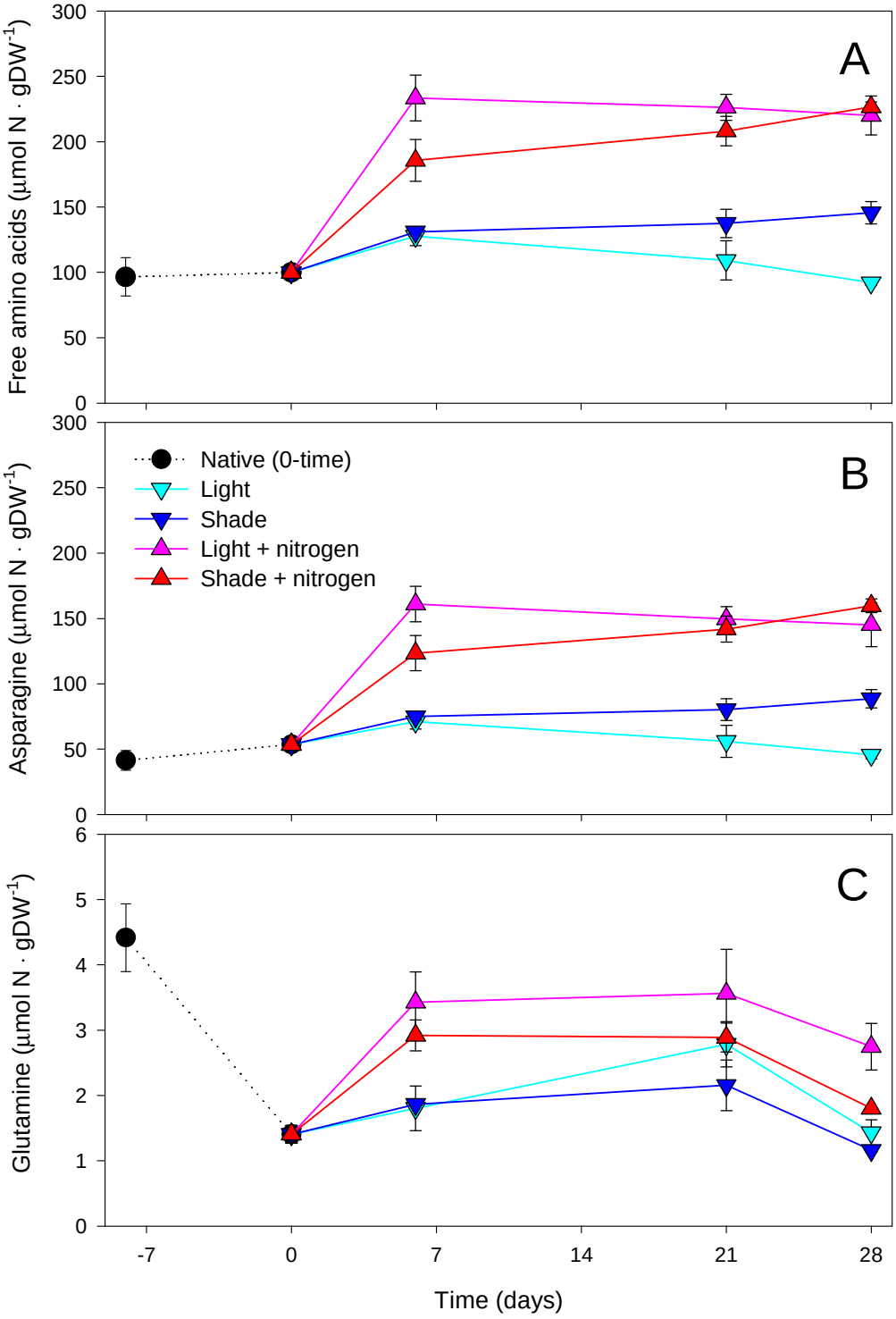



**Figure 3.5.** Changes in (**A**) total free amino acid, (**B**) asparagine and (**C**) glutamine content in *Ulva pertusa* in culture chambers maintained under either ambient or shaded light, with and without nitrogen addition. Values are means ± standard errors for four separate replicates.

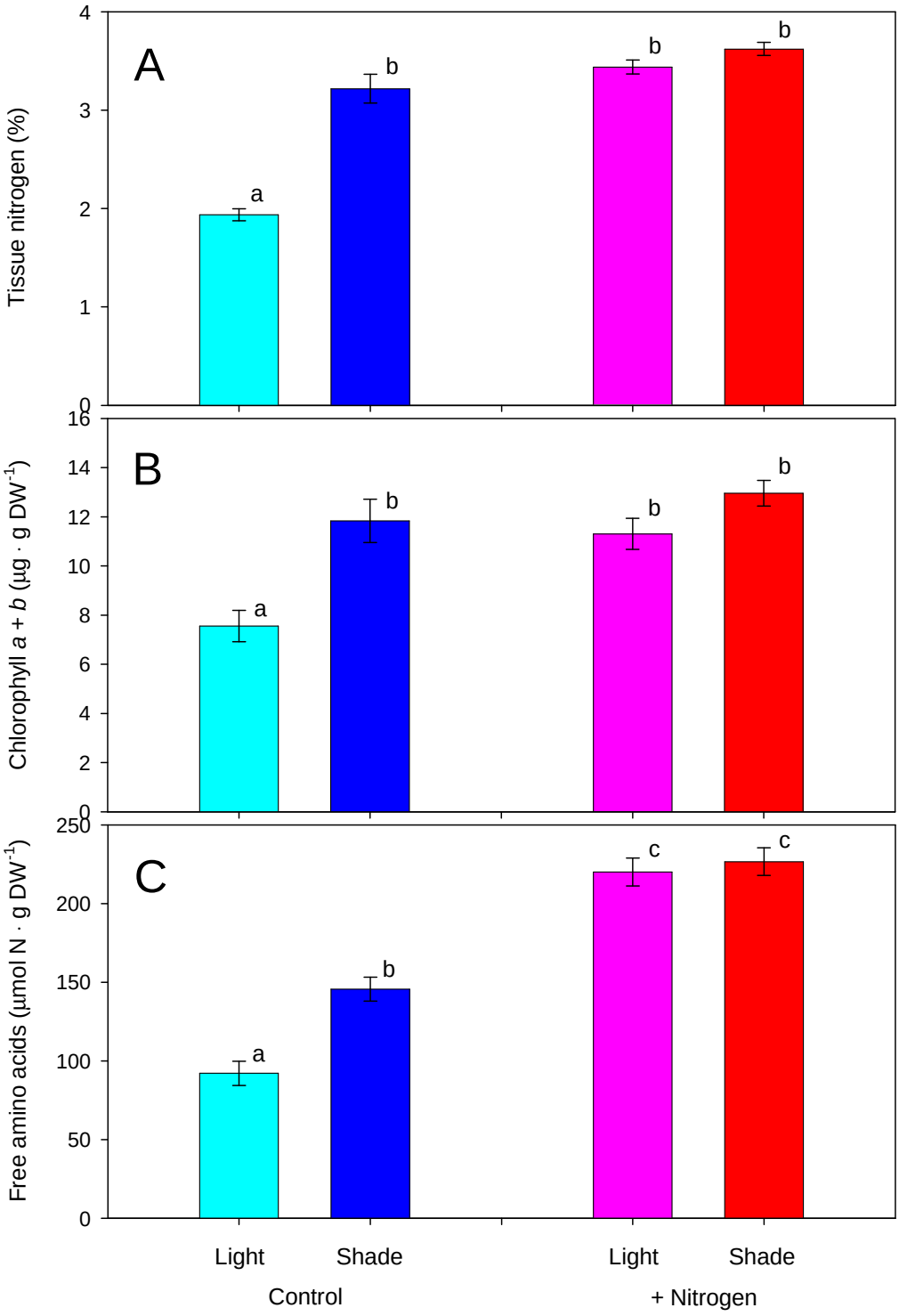



**Figure 3.6.** Effect of light and nitrogen addition on (**A**) tissue nitrogen, (**B**) total chlorophyll and (**C**) free amino acid content in *Ulva pertusa* after 28 days. Values are means ± standard errors for four separate replicates. Bars labelled with the same lower case letter do not differ significantly ($P > 0.05$) according to the Holm-Sidak method for pair-wise multiple comparisons.

Rates of ammonium assimilation were also compared at the end of the experiment (day 28). The rate of ammonium assimilation in *Ulva* was lower in both shaded treatments and lowest (2.7 ± 1.9 μmol · g $DW^{-1}$ · $h^{-1}$) overall in the shaded, nitrogen enriched plants (Figure 3.7). The highest rate of assimilation (31.6 ± 7.9 μmol · g $DW^{-1}$ · $h^{-1}$) was found in the non-enriched, light treated *Ulva*. Two-way analysis of variance showed that there was a significant effect of both light and nitrogen addition on ammonium assimilation in *Ulva* (Table 3.2).

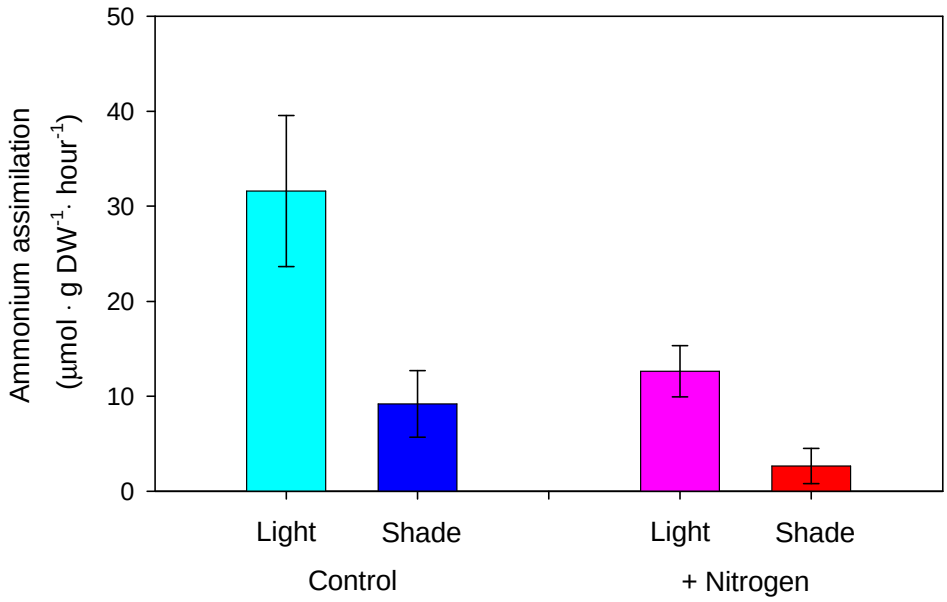



**Figure 3.7.** Rate of ammonium assimilation in *Ulva pertusa* maintained under either ambient or shaded light, with and without nitrogen addition for 28 days. Values are means ± standard errors for four separate replicates.

**Table 3.2.** Two-way analysis of variance of the rate of ammonium assimilation for *Ulva pertusa* maintained under either ambient or shaded light, with and without nitrogen addition for 28 days.

**Two-way ANOVA on differences in rate of ammonium assimilation in *Ulva***

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 898.83 | 898.83 | 9.29 | 0.012 |
| Nitrogen | 1 | 557.70 | 557.70 | 5.76 | 0.037 |
| Light × Nitrogen | 1 | 132.59 | 132.59 | 1.37 | 0.269 |
| Residual | 10 | 967.72 | 96.77 | | |
| Total | 13 | 2679.01 | 206.08 | | |

Natural abundance stable nitrogen isotopes were also compared at the end of the experiment. *Ulva* tissue $\delta^{15}N$ values from the nitrogen-enriched treatments were clearly different to those in *Ulva* from the unenriched controls (i.e., those supplied with natural seawater) (Figure 3.8). In addition, while there was a significant effect of nitrogen enrichment ($F_{1,13} = 4290$, $P < 0.001$) in *Ulva*, there was no significant effect on $\delta^{15}N$ values by light treatment ($F_{1,13} = 0.04$, $P = 0.84$). The average $\delta^{15}N$ values for unenriched controls for light and shaded treatments were 8.0 ± 0.0 and 8.0 ± 0.1, respectively. Conversely, the average $\delta^{15}N$ values for enriched *Ulva* from light and shaded treatments were -8.1 ± 0.1 and -8.0 ± 0.6, respectively (i.e., *Ulva* tissue $\delta^{15}N$ values were clearly influenced by the $\delta^{15}N$ of synthetic nitrogen ($NH_4Cl$)). However, a difference between tissue $\delta^{15}N$ in enriched *Ulva* and that of the synthetic nitrogen source (–5.5 ‰) suggested a fractionation in *Ulva* tissue of around –2.5 ‰ (Figure 3.8).

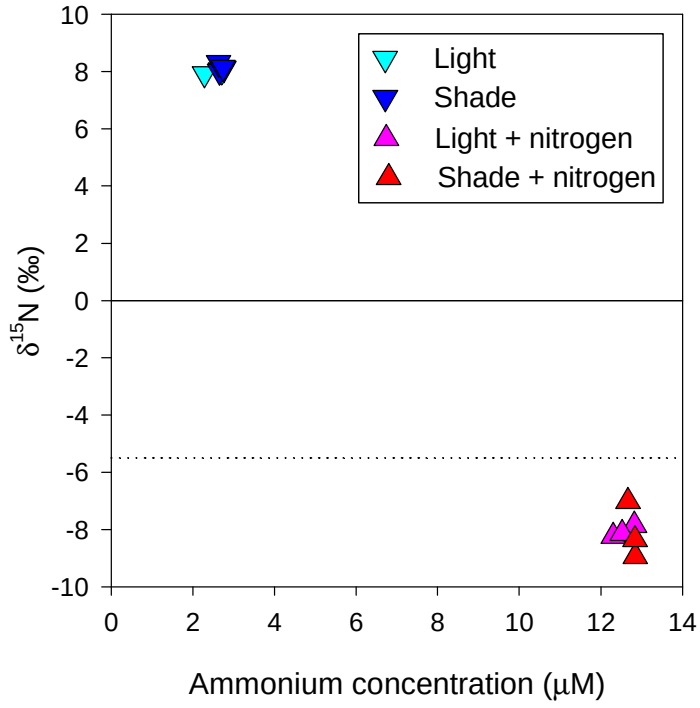



**Figure 3.8.** Change in tissue $\delta^{15}N$ with change in ammonium concentration (added as ammonium cloride ($NH_4Cl$)) in *Ulva pertusa* in culture chambers maintained under either ambient or shaded light, with and without nitrogen addition for 28 days. The dashed line represents the $\delta^{15}N$ signature (-5.5 ‰) of synthetic ammonium cloride.

**Experiment 2. Effect of nitrogen concentration on FAA content in *Ulva pertusa*. August / September (late winter) 2003.**

Changes in free amino acids (FAA) in *Ulva* were examined in response to changes in nitrogen concentration. The total FAA pool (dominated by asparagine) increased linearly with increases in ammonium addition at lower concentrations (between 2 μM to around 7 μM) (Figure 3.9). However the total FAA pool began to saturate at higher concentrations (> 15 μM) reaching a plateau at about 340 μmol N · g $DW^{-1}$ (Figure 3.9). Asparagine showed a similar response reaching a plateau at about 240 μmol N · g $DW^{-1}$ although at slightly lower concentrations of around 10 - 12 μM (Figure 3.9, B). From the lowest nitrogen concentration of close to 2 μM (i.e., natural seawater with no added nitrogen) to the highest concentration of 40 μM the FAA pool ranged from about 30 μmol N · g $DW^{-1}$ to 347 μmol N · g $DW^{-1}$ representing a 12-fold range in values. Asparagine ranged from 13 μmol N · g $DW^{-1}$ to 250 μmol N · g $DW^{-1}$ representing a 16-fold range of values.

Glutamate and glutamine (key amino acids involved in the glutamine synthetase [GS] / glutamate synthase [GOGAT] pathway) showed quite different relationships to each other with increases in ammonium concentration. Both these amino acids were present in *Ulva* at lower levels than asparagine and ranged from 1.9 ± 0.2 to 6.0 ± 0.1 μmol N · g DW-1 and 3.5 ± 0.3 to 9.0 ± 0.6 μmol N · g DW-1 for glutamine and glutamate, respectively. Similarly to asparagine, glutamine increased initially with increases in ammonium concentration but then began to saturate at ammonium concentrations above about 10 μM (Figure 3.10, A). Moreover, asparagine also showed a strong linear (R2 = 0.96) correlation with glutamine (data not shown). In contrast to the trend in glutamine, glutamate increased with ammonium concentration up to about 10 μM and then declined as concentrations increased beyond this value (Figure 3.10, B). Compared to the changes that occurred in individual and total free amino acids at lower ammonium concentrations (i.e. up to about 10 μM), the ratio of glutamine : glutamate remained relatively constant at values of around 0.27 at these lower ammonium concentrations and then showed a gentle linear increase with higher concentrations (Figure 3.10, C). For each concentration, the percentage contribution of each individual amino acid to the total FAA pool was calculated. The mean percentage contribution of individual amino

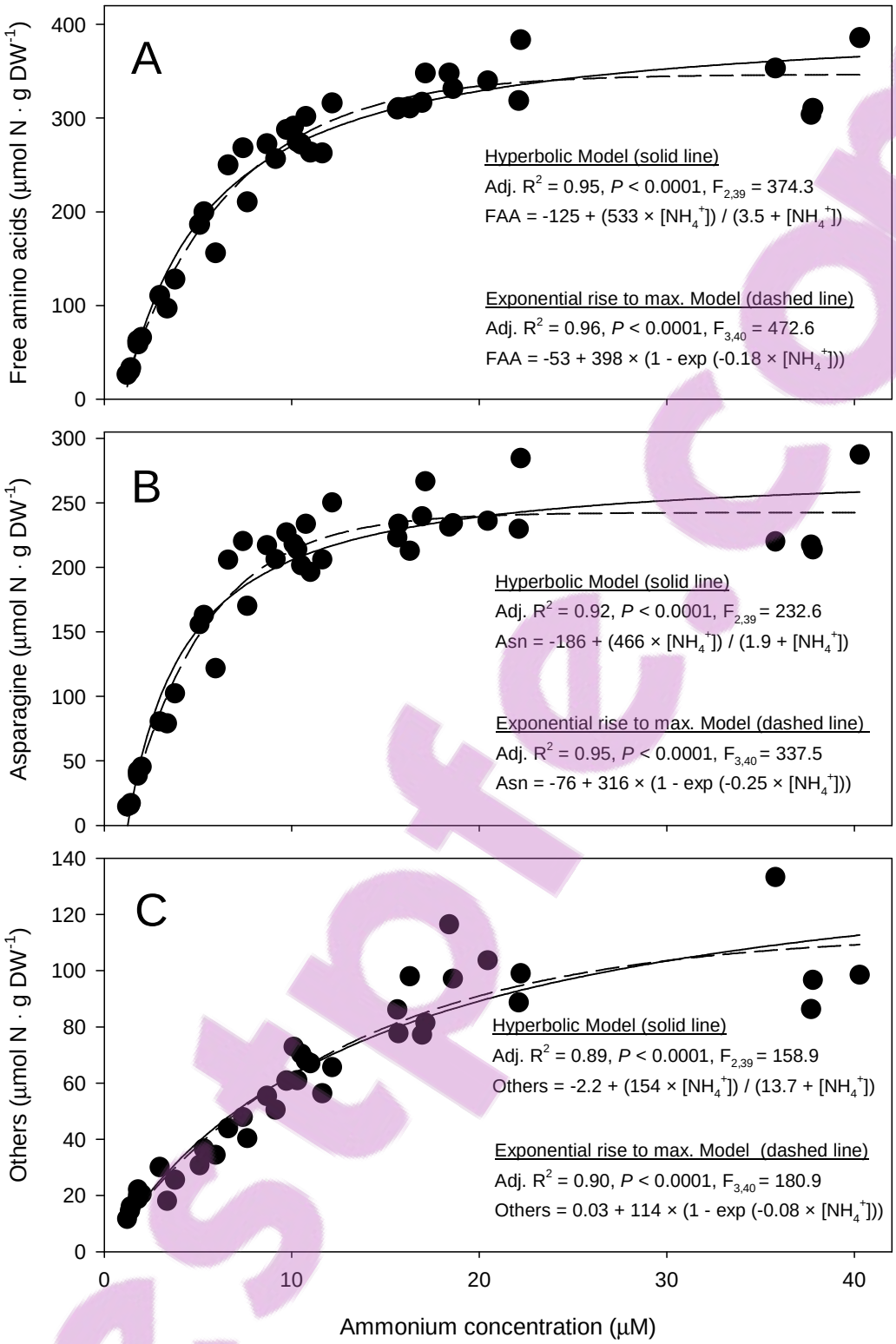



**Figure 3.9.** Change in (**A**) total free amino acids, (**B**) asparagine and (**C**) total amino acids minus asparagine content in *Ulva pertusa* with change in ammonium concentration.

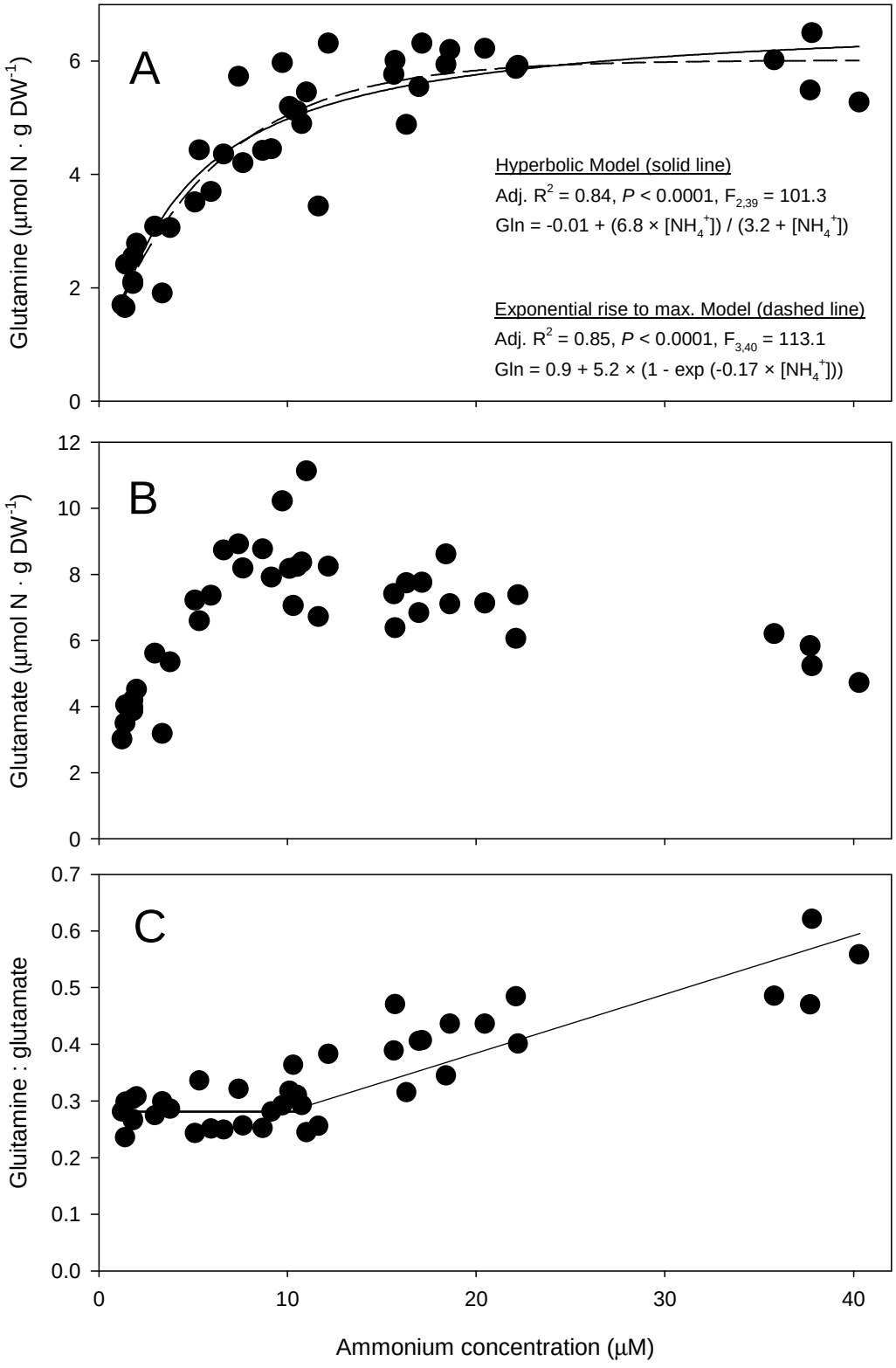



**Figure 3.10.** Change in (**A**) glutamine, (**B**) glutamate and (**C**) glutamine : glutamate content in *Ulva pertusa* with change in ammonium concentration. Note that in the last plot (**C**) a suggested relationship is fitted by eye.

**Table 3.3.** Contribution of individual amino acids to the total free amino acid pool, and correlation coefficients between individual amino acids and the total free amino acid pool. *P*-values are indicated in bold text. Note that a negative symbol for $R^2$ values indicates a negative slope relationship.

| Amino acid | Contributution in N to % FAA | Cummulative % FAA | $R^2$ | *P*-value |
|---|---|---|---|---|
| Asparagine | 73.43 | 73.43 | 0.98 | **0.0000** |
| Histidine | 6.85 | 80.28 | 0.79 | **0.0001** |
| Glutamate | 3.93 | 84.21 | 0.50 | **0.0037** |
| U2 $^{9.48}$ | 3.47 | 87.68 | 0.85 | **0.0000** |
| Proline | 3.21 | 90.89 | 0.67 | **0.0012** |
| Glutamine | 2.42 | 93.31 | 0.99 | **0.0004** |
| Serine | 1.38 | 94.70 | 0.87 | **0.0000** |
| Alanine | 1.03 | 95.72 | 0.57 | **0.0017** |
| Glycine/Citrulline | 0.99 | 96.71 | 0.65 | **0.0046** |
| Aspartate | 0.85 | 97.56 | 0.89 | **0.0000** |
| Valine | 0.56 | 98.13 | 0.49 | **0.0115** |
| Methionine | 0.47 | 98.59 | 0.43 | **0.0206** |
| Phenylalanine | 0.38 | 98.97 | -(0.24) | **0.0000** |
| Thrionine | 0.38 | 99.35 | 0.94 | 0.1110 |
| Leucine | 0.29 | 99.65 | 0.13 | 0.2660 |
| IsoLeucine | 0.22 | 99.87 | 0.27 | 0.0897 |
| Taurine | 0.13 | 100.00 | 0.52 | **0.0080** |

acids was then calculated for all concentrations (Table 3.3). Of the 17 amino acids examined in Ulva, the most dominant (in terms of μmol N equivalents) in order were: asparagine, histidine, glutamate, an unknown amino compound $U2^{9.48}$, proline, and glutamine. Together these 6 amino acids contributed 93 % of the total FAA pool. In addition, most amino acids except phenylalanine, leucine and isoleucine significantly correlated with the total FAA pool (Table 3.3).

Ranges in values of TChl, TN and growth were compared over the range of concentrations examined (note that TN was only measured in Experiment 2a and was therefore compared to a concentration range of 1.3 ± 0.1 μM to 21.6 ± 0.6 μM). Compared with the 12-fold range of FAA values, TChl ranged from 3.7 ± 0.2 mg · g $DW^{-1}$ to 9.9 ± 0.1 mg · g $DW^{-1}$, representing only a 2.6-fold range (data not shown). However, it should also be noted that these experiments only ran for 10 days and it was evident from Experiment 1 that chlorophyll levels would probably have

increased further with more time. TN showed a similar 2.4-fold range in values (1.4 to 3.9 %) (Figure 3.11, A). Decreasing values of $\delta^{15}N$ in *Ulva* tissue indicated an increasing contribution of the synthetic nitrogen source (–5.5 ‰) resulting in a shift away from the $\delta^{15}N$ signature (of around 8 ‰) seen in natural un-enriched *Ulva* (Figure 3.11, B).

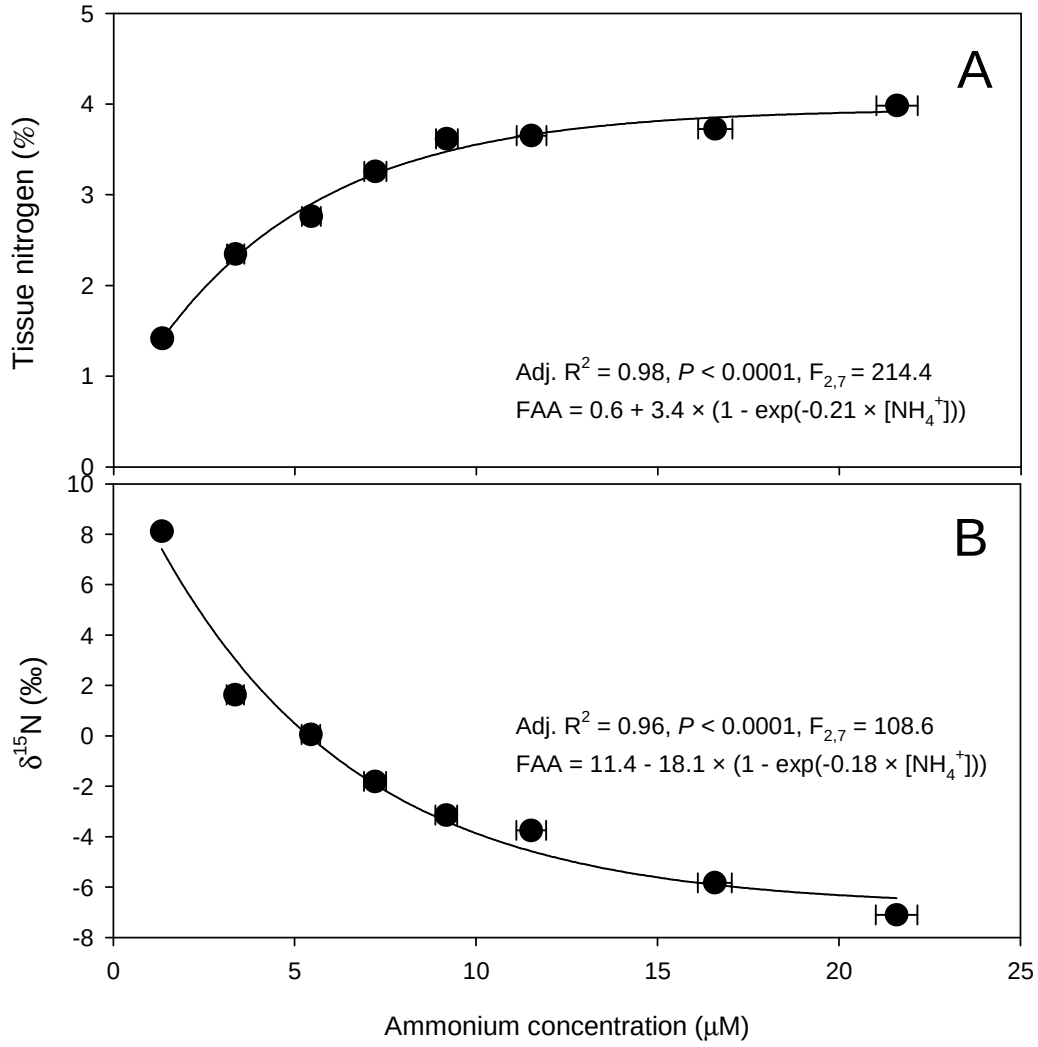



**Figure 3.11.** Change in (**A**) tissue nitrogen and (**B**) $\delta^{15}N$ content in *Ulva pertusa* with change in ammonium concentration. Values are from three composited replicates from Experiment 2a only.

Compared with nitrogen indices there was no significant ($F_{1,11}$ = 1.44, $P$ = 0.26) effect of nitrogen concentration on growth rate with *Ulva* for all concentrations

showing average growth rates of around 0.12 ± 0.00 day$^{-1}$ (data not shown). However at this growth rate *Ulva* biomass well over doubled during the enrichment phase of the experiments suggesting that significant tissue turnover should have occurred over the duration of the experiment.

**Experiment 3. Effect of flow rate and water motion on growth and N-indices in *Ulva pertusa*. March / April (late summer) 2004.**

There was a significant effect of both average bulk water velocity through growth chambers and the addition of turbulence on growth rate in *Ulva* (Figure 3.12). At higher flow rates the addition of turbulence close to doubled the growth rate of *Ulva*. The highest growth rate observed in this experiment was 0.20 day$^{-1}$ in chambers with the highest average bulk water velocity and the addition of turbulence (Figure 3.12). Growth rates in both the turbulent and non-turbulent treatments appeared to reach plateaux at the higher flow rates between 40 and 60 cm · min$^{-1}$.

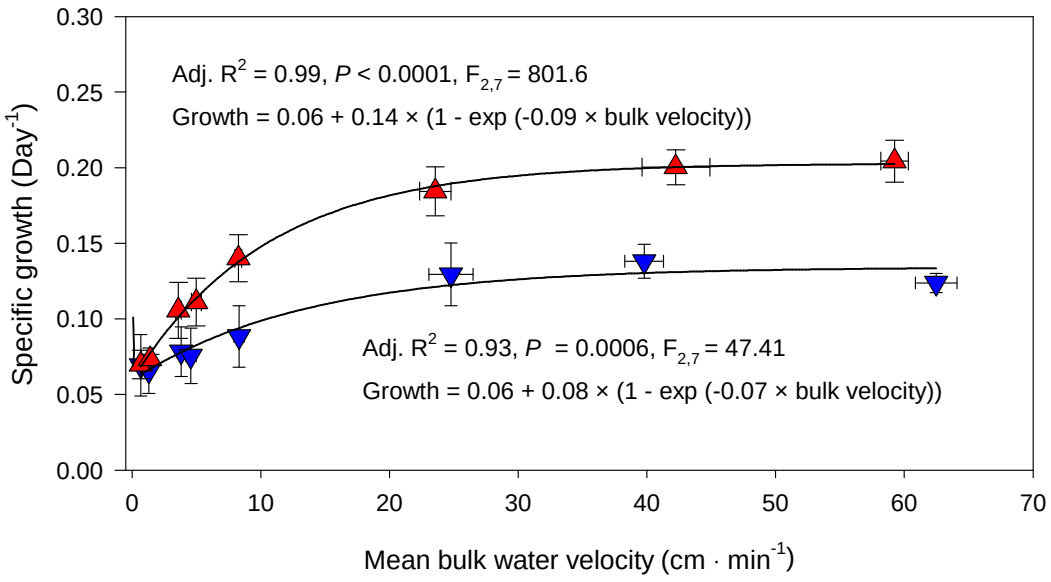



**Figure 3.12.** Change in growth in *Ulva* with change in average bulk flow rate and the addition of turbulence (i.e., with the presence of a dump bucket). Red symbols are values for turbulent flow and blue symbols are values for non-turbulent flow. Regression statistics are arranged adjacent to their respective fitted regression curves. Values are means ± standard errors for three separate replicates.

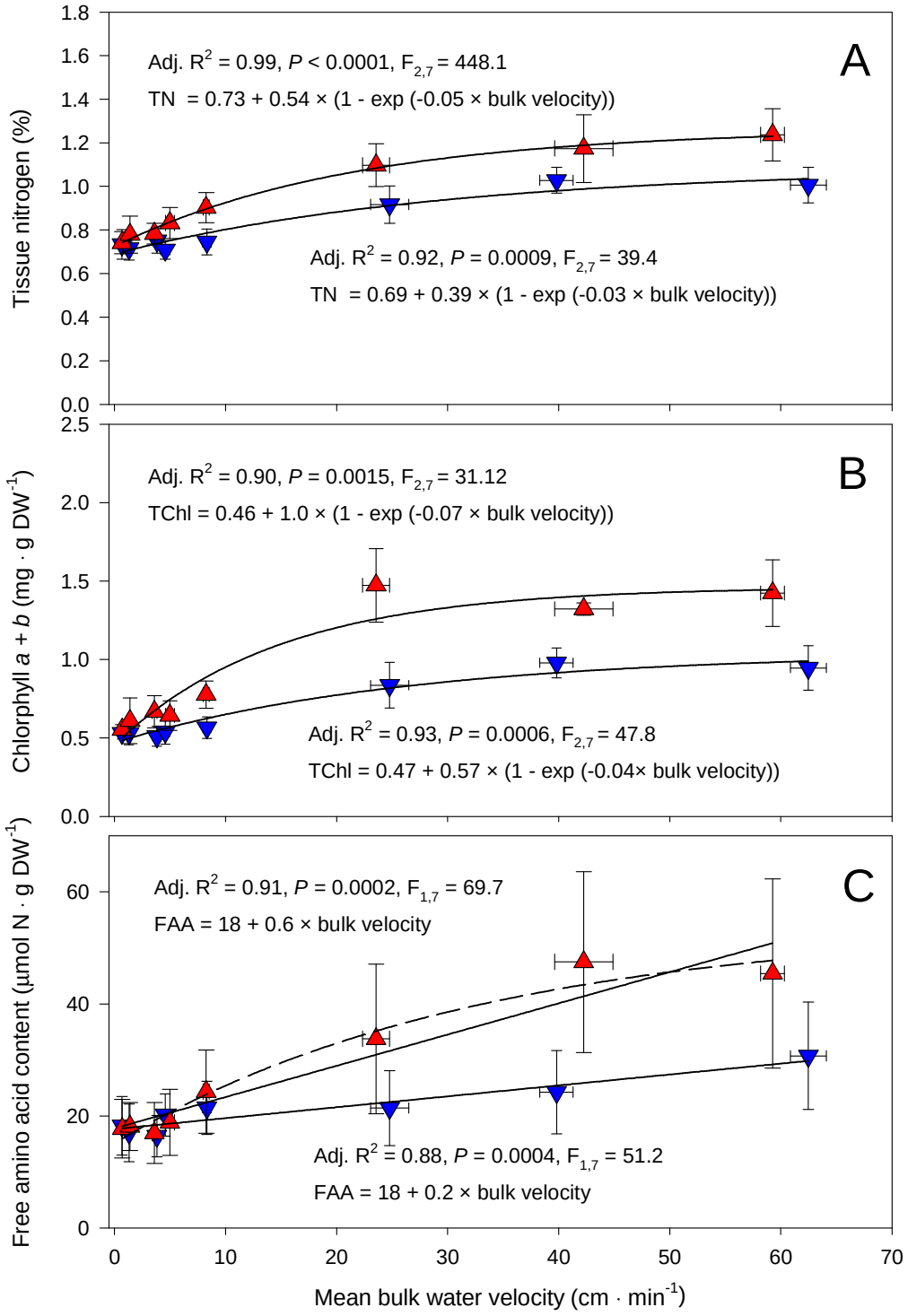



**Figure 3.13.** Change in (**A**) total nitrogen content, (**B**) chlorophyll and (**C**) free amino acid content in *Ulva* with change in average bulk flow rate and the addition of turbulence (i.e., with the addition of a dump bucket). Red symbols are values for turbulent flow and blue symbols are values for non-turbulent flow. Regression statistics are arranged adjacent to their respective fitted regression curves. Note that the dashed line in plot **C** represents a suggested non-linear, exponential rise to maximum relationship. Values are means ± standard errors for three separate replicates.

Similar effects of water motion on TN, TChl and FAA content were also observed (Figure 3.13, A, B and C, respectively), although differences were generally less pronounced than differences in growth rate. Interestingly, increases in FAA content as well as being more linear with respect to flow rate showed slightly larger average differences between turbulent and non-turbulent treatments compared with TN content. However, there was also more temporal variability in FAA content between replicates runs (Figure 3.13, C) which may have related to a decline in N-indices (through time) of the stock *Ulva* that was used for the three trials. It was noted that TN content in the starting material was successively lower at the beginning of each trial.

**Experiment 4. Effect of light, and ammonium versus nitrate addition on growth and N-indices in *Ulva pertusa*. January (summer) 2005.**

Growth rate in *Ulva* showed a similar overall contrast between unshaded and shaded treatments and was consistent with results from the first experiment (Experiment 1) (Figure 3.14). There was a significant effect of light throughout this experiment. In addition, there was a statistically significant effect of nitrogen source (ammonium versus nitrate) and time, on growth (Figure 3.14 and Table 3.4). However, by the third growth period there was no significant effect of nitrogen source on growth rate ($F_{1,11}$ = 0.11, $P$ = 0.749). According to three-way ANOVA output there was no significant interaction between light, nitrogen source and time (Table 3.4). Although not included in the statistical comparison below it is worth noting that growth in the unenriched, light-saturated treatment declined markedly during the last growth period and presumably reflected a nitrogen (or phosphorus) limitation (Figure 3.14).

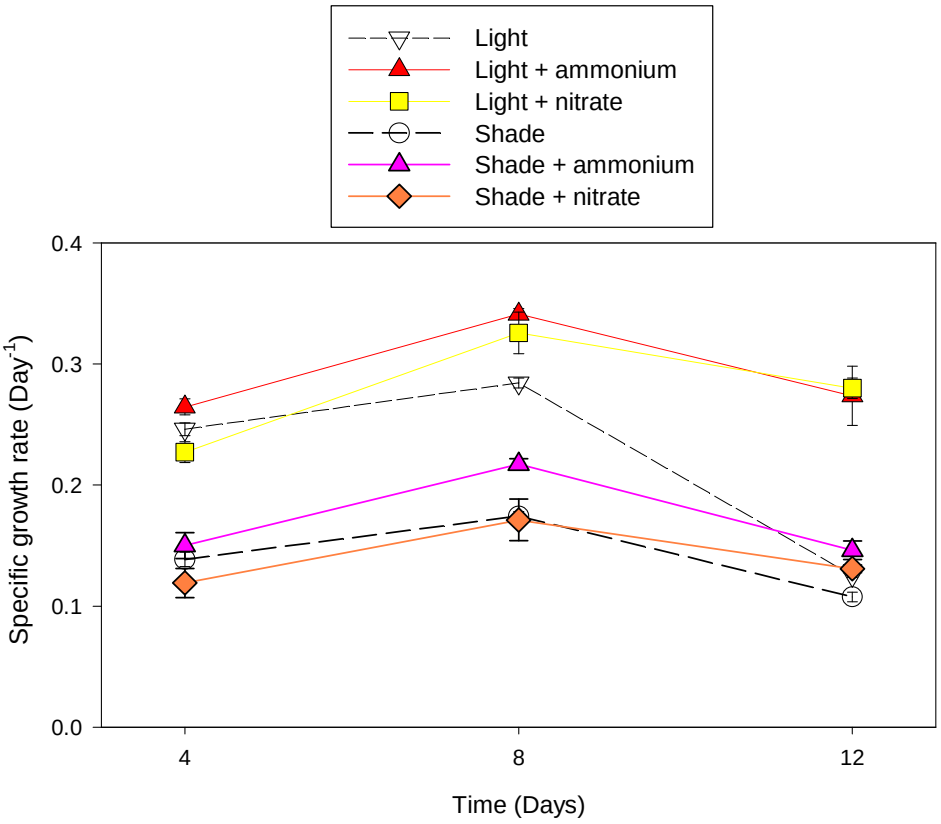



**Figure 3.14.** Effect of nitrogen source (ammonium versus nitrate) on specific growth rate in *Ulva pertusa*. For enriched treatments values are means ± standard errors for three separate replicates.

**Table 3.4.** Three-way analysis of variance of growth in *Ulva pertusa* over 12 days maintained under either ambient or shaded light, with either ammonium or nitrate addition.

**There-way ANOVA on differences in growth rate in *Ulva***

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 0.151 | 0.151 | 345.60 | <0.001 |
| Nitrogen | 1 | 0.005 | 0.005 | 11.03 | 0.003 |
| Time | 2 | 0.036 | 0.018 | 40.63 | <0.001 |
| Light x Nitrogen | 1 | 0.001 | 0.001 | 1.19 | 0.285 |
| Light x Time | 2 | 0.002 | 0.001 | 1.74 | 0.197 |
| Nitrogen x Time | 2 | 0.002 | 0.001 | 1.81 | 0.186 |
| Light x Nitrogen x Time | 2 | 0.001 | 0.000 | 0.64 | 0.537 |
| Residual | 24 | 0.011 | 0.000 | | |
| Total | 35 | 0.206 | 0.006 | | |

Similarly to Experiment 1 the effects of nitrogen enrichment were visually apparent in the colour of *Ulva* thalli. However, compared to the winter experiment there was more contrast between *Ulva* from enriched and control treatments (Appendix 3.1). At the end of the experiment while there was no significant effect of nitrogen source (i.e., ammonium versus nitrate addition) on both TN and TChl content in *Ulva*, there was a significant effect of light (Table 3.5). *Ulva* in shaded treatments had higher TN and TChl content (Figure 3.15, A and B, respectively). Conversely, there was no effect of either nitrogen source or light level on FAA content in *Ulva* (Figure 3.15, C and Table 3.5). However, it is worth noting that total FAA values measured midway through the experiment, while not significantly different between light treatments ($F_{1,11}$ = 2.7, $P$ = 0.14), were significantly higher in ammonium-enriched treatments compared with nitrate-enriched treatments ($F_{1,11}$ = 55.4, $P$ < 0.001) (data not shown). This suggests that there was an initial limitation on the utilisation of nitrate, and may have been linked to initial growth rates being lower in nitrate-supplied *Ulva*.

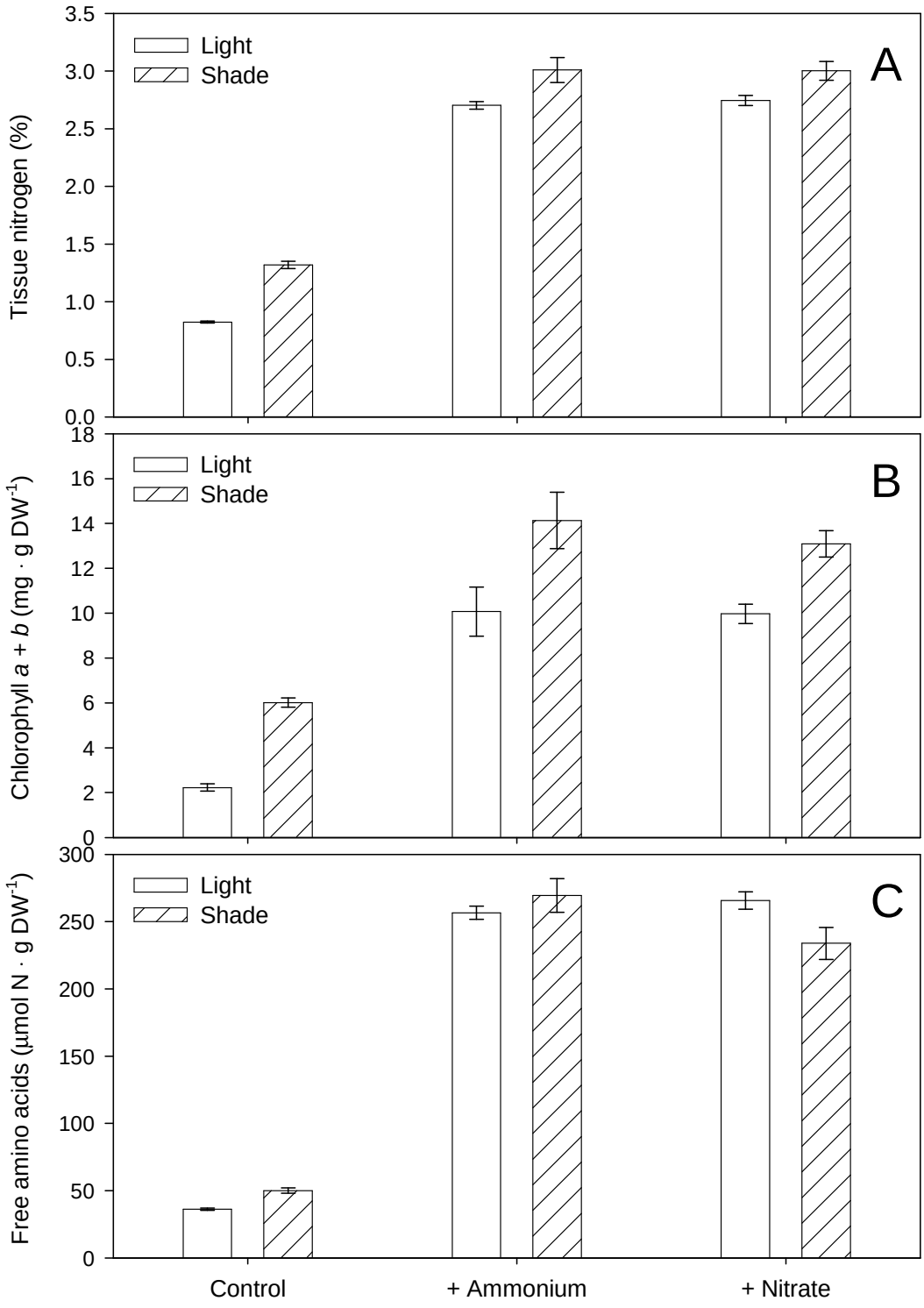



**Figure 3.15.** Effect of nitrogen source (ammonium versus nitrate) on (**A**) tissue nitrogen, (**B**) chlorophyll and (**C**) free amino acid content in *Ulva pertusa*. Except for controls (comprised of two pseudo replicates and one true replicate) values are means ± standard errors for three separate replicates.

**Table 3.5**. Two-way analysis of variance in (**A**) total tissue nitrogen content, (**B**) total chlorophyll and (**C**) free amino acid content in *Ulva pertusa* maintained under contrasting levels of light and nitrogen supplied as either nitrate or ammonium.

**A) Two-way ANOVA on differences in *Ulva* total tissue nitrogen content**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 0.2390 | 0.2390 | 14.84 | 0.005 |
| N-source | 1 | 0.0009 | 0.0009 | 0.05 | 0.821 |
| Light x N-source | 1 | 0.0018 | 0.0018 | 0.11 | 0.747 |
| Residual | 8 | 0.1290 | 0.0161 | | |
| Total | 11 | 0.3700 | 0.0337 | | |

**B) Two-way ANOVA on differences in *Ulva* total chlorophyll content**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 28.622 | 28.622 | 14.31 | 0.005 |
| N-source | 1 | 0.838 | 0.838 | 0.42 | 0.536 |
| Light x N-source | 1 | 2.189 | 2.189 | 1.09 | 0.326 |
| Residual | 8 | 16.006 | 2.001 | | |
| Total | 11 | 47.655 | 4.332 | | |

**C) Two-way ANOVA on differences in *Ulva* total free amino acid content**

| Source of Variation | *DF* | *SS* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Light | 1 | 264.8 | 264.8 | 0.73 | 0.417 |
| N-source | 1 | 526.3 | 526.3 | 1.45 | 0.263 |
| Light x N-source | 1 | 1501.9 | 1501.9 | 4.15 | 0.076 |
| Residual | 8 | 2898.2 | 362.3 | | |
| Total | 11 | 5191.2 | 471.9 | | |

Consistent with the previous experiments, asparagine was the dominant amino acid under nitrogen enrichment and was not significantly affected by either light ($F_{1,11}$ = 1.9, $P$ = 0.21) or nitrogen source ($F_{1,11}$ = 1.5, $P$ = 0.25), although there were slightly higher levels in the shaded, ammonium-enriched *Ulva* (Figure 3.16, A). Similarly, histidine was not significantly affected by light ($F_{1,11}$ = 3.5, $P$ = 0.096), or nitrogen source ($F_{1,11}$ = 0.036, $P$ = 0.85) (Figure 3.16, B). Some amino acids, however, did show different responses to either light level and / or nitrogen source. Glutamine and glutamate levels were significantly ($F_{1,11}$ = 35.5, $P$ < 0.001 and $F_{1,11}$ = 61.1, $P$ < 0.001, respectively) affected by light, but not nitrogen source ($F_{1,11}$ = 0.02, $P$ = 0.89 and $F_{1,11}$ = 0.55, $P$ = 0.48 for glutamine and glutamate, respectively) (Figure 3.16, C

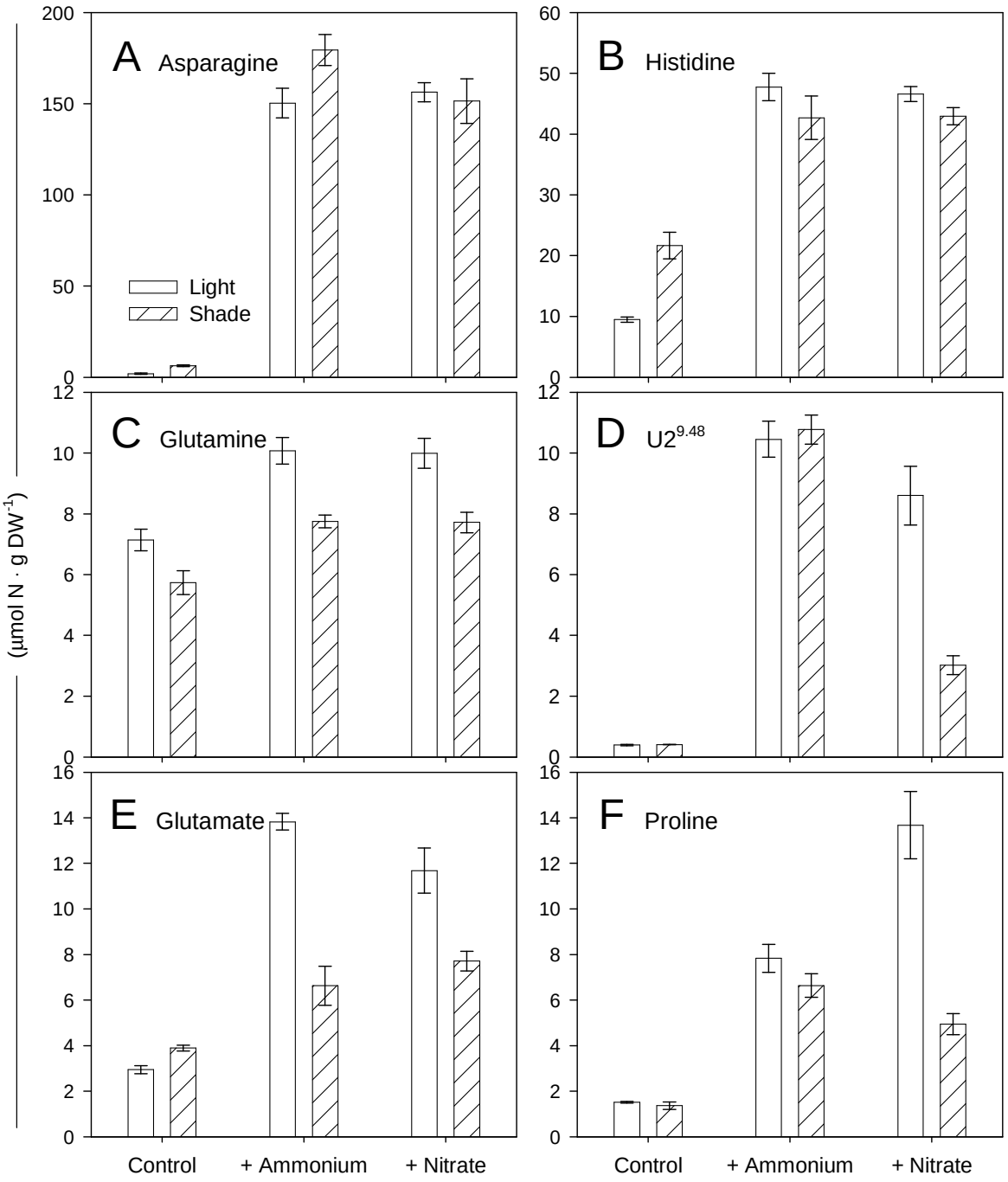



**Figure 3.16.** Effect of nitrogen source (ammonium versus nitrate) on (**A**) asparagine, (**B**) histidine, (**C**) glutamine, (**D**) $U2^{9.48}$, (**E**) glutamate and (**F**) proline content in *Ulva pertusa*. Except for controls (comprised of two pseudo replicates and one true replicate) values are means ± standard errors for three separate replicates.

and E). Levels of proline and U2$^{9.48}$, on the other hand, were significantly affected by both light ($F_{1,11}$ = 32.37, $P$ < 0.001 and $F_{1,11}$ = 17.25, $P$ = 0.003, respectively) and nitrogen source ($F_{1,11}$ = 5.64, $P$ = 0.045 and $F_{1,11}$ = 57.46, $P$ < 0.001, respectively) (Figure 3.16, D and F). Both proline and U2$^{9.48}$ showed higher levels under unshaded, nitrate-enrichment compared with their shaded counterparts (Figure 3.16, D and F).

Results for rates of ammonium assimilation in *Ulva* were similar to results from Experiment 1 (conducted in the winter) with higher rates in unshaded treatments compared with shaded. However, values were generally considerably higher in this experiment (conducted in the summer). Values ranged from about 68 μmol · g DW$^{-1}$ · h$^{-1}$ in both shaded nitrogen (ammonium and nitrate) enriched treatments to 141.4 ± 12.1 μmol · g DW$^{-1}$ · h$^{-1}$ in unshaded ammonium-enriched *Ulva* (Figure 3.17). There was a significant difference ($P$ = 0.044) in the rate of ammonium assimilation between unshaded ammonium-enriched and unshaded nitrate-enriched *Ulva* (Figure 3.17).

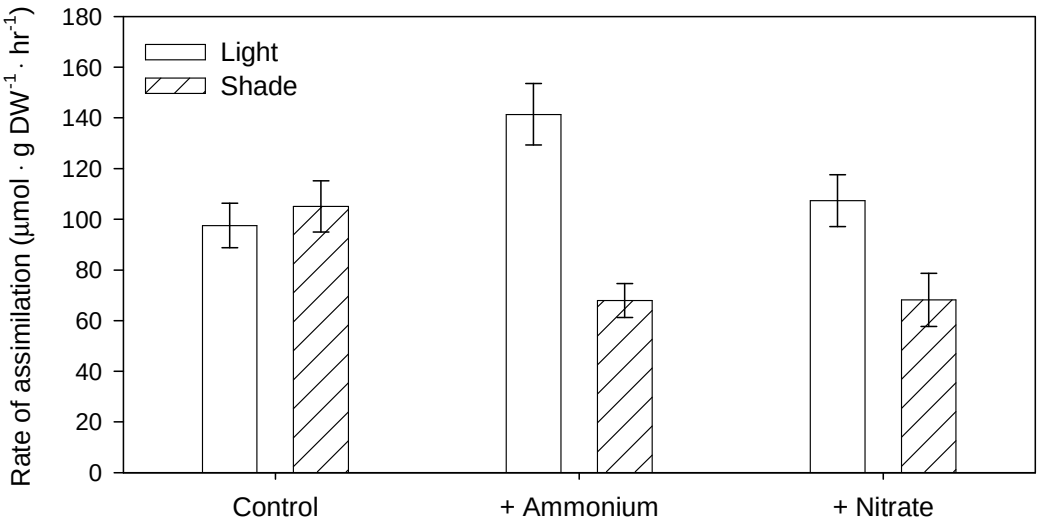



**Figure 3.17.** Effect of nitrogen source (ammonium versus nitrate) on rate of ammonium assimilation in *Ulva pertusa*. Except for controls (comprised of two pseudo replicates and one true replicate) values are means ± standard errors for three separate replicates.

Natural abundance stable nitrogen isotopes in *Ulva* supplied with unenriched natural seawater had $\delta^{15}N$ values of 7.3 ± 0.0 ‰ and 7.6 ± 0.1 ‰ for unshaded and shaded treatments, respectively (Figure 3.18, A). For ammonium-enriched *Ulva* $\delta^{15}N$ values were -4.2 ± 0.1 ‰ and -7.4 ± 0.4 ‰ for unshaded and shaded treatments, respectively, and for nitrate-enriched *Ulva* 3.8 ± 0.0 ‰ and 3.8 ± 0.1 ‰ for unshaded and shaded treatments, respectively (Figure 3.18, A). Fractionation values were calculated for enriched *Ulva* using source values for synthetic ammonium (-5.5 ‰) and for nitrate (3.95 ‰). For *Ulva* in natural seawater an estimate from Gartner (2002) for $\delta^{15}N$ values in natural seawater of 6.7 ‰ was used. Fractionation of $^{15}N$

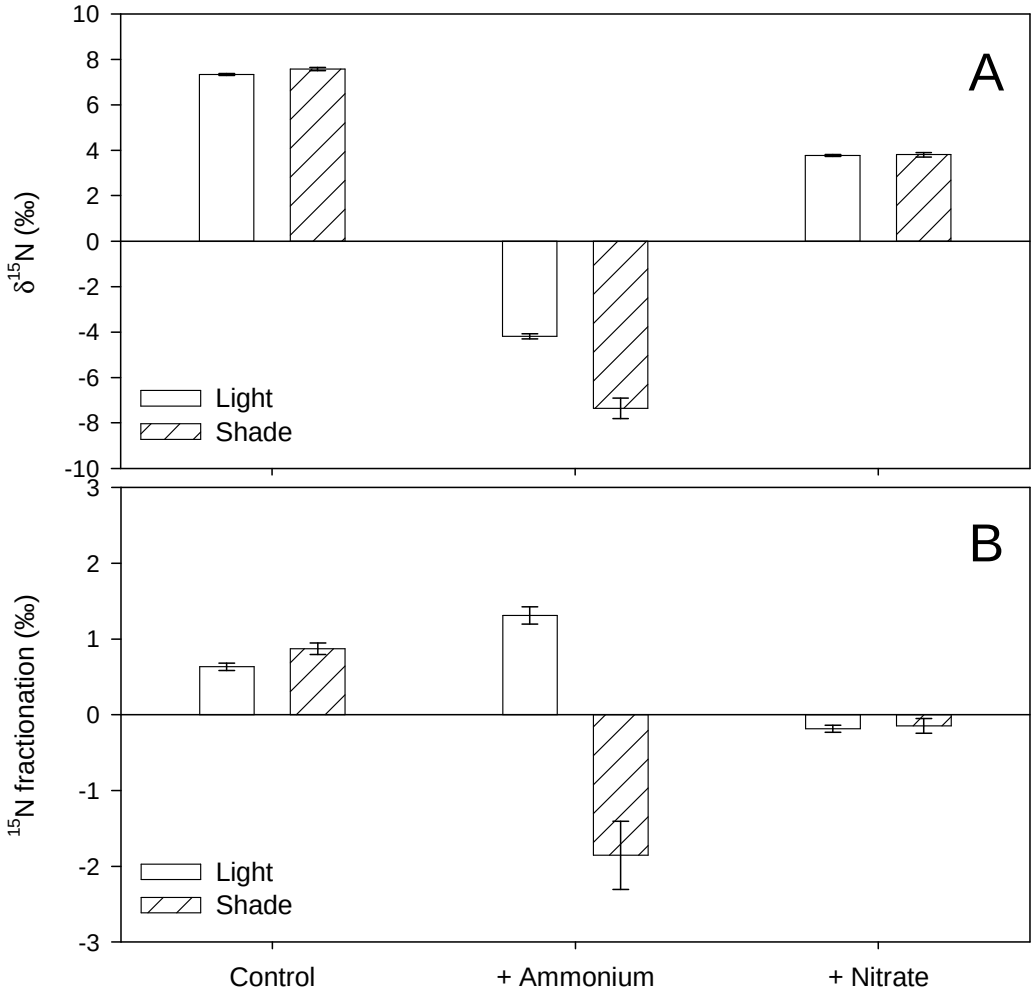



**Figure 3.18.** Effect of nitrogen source (ammonium versus nitrate) on (**A**) $\delta^{15}N$ values and (**B**) $^{15}N$ fractionation in *Ulva pertusa*. Except for controls (comprised of two pseudo replicates and one true replicate) values are means ± standard errors for three separate replicates.

in *Ulva* supplied with natural seawater was similar for both unshaded and shaded treatments at values of 0.6 ± 0.0 ‰ and + 0.9 ± 0.1 ‰, respectively (Figure 3.18, B). For ammonium-enriched *Ulva* $^{15}N$ fractionation values were 1.3 ± 0.1 ‰ and -1.9 ± 0.4 ‰ for unshaded and shaded treatments, respectively. However, for nitrate-enriched *Ulva* $^{15}N$ fractionation values for unshaded and shaded treatments were both negative but small at -0.2 ± 0.0 ‰ and -0.1 ± 0.1 ‰, respectively (Figure 3.18, B).

**Summary**

***Nitrogen indices and seawater nitrogen concentration***

Overall performance of nitrogen indices in *Ulva* was examined by combining the results for all four experiments. With the exception of Experiment 3 (in which no nitrogen was added) the addition of nitrogen resulted in significant changes in nearly all N-indices examined. From pooled data of these experiments, which compared responses to un-enriched natural seawater with those in 10 μM enriched seawater (irrespective of nitrogen source and light treatment), the most pronounced biochemical response to external nitrogen concentration was that of amino acids (Table 3.6). While the largest proportional change (22-fold) was shown by $U2^{9.48}$ the largest quantitative change was seen in asparagine (Asn) (Table 3.6). *Ulva* in nitrogen enriched seawater had Asn levels of 173 μmol N · g $DW^{-1}$ compared to 25.4 μmol N · g $DW^{-1}$ in natural un-enriched seawater. This represented a 6.8-fold increase in response to a 5.5-fold increase in seawater nitrogen concentration (Table 3.6). The FAA acid pool in *Ulva* showed a slightly lower 4.4-fold increase in response to an increase in seawater nitrogen (Table 3.6). Aspartate (Asp), glutamine (Gln) and glutamate (Glu) all showed minor increases in enriched *Ulva* although the difference in glutamine levels was not significant (Table 3.6). The sum of the remaining (other) amino acids also showed a significant (2.7-fold) increase with an increase in seawater nitrogen. Total tissue nitrogen (TN) and chlorophyll *a* and *b* also increased with the addition of nitrogen. However their responses to nitrogen addition were less pronounced (around 2-fold) compared with changes in the FAA pool. The ratios of asparagine : aspartate and total free amino acid-N (FAA-N) : total tissue nitrogen (TN) increased significantly with increases in seawater nitrogen concentration whereas the ratio of glutamine : glutamate was not significantly affected (Table 3.6).

**Table 3.6.** Combined results from four experiments of changes in biochemical N-indices to 10 µM inorganic nitrogen addition compared with natural seawater controls (irrespective of light treatment). Significant differences (according to t-tests) are indicated in bold text. Proportional changes in the enriched *Ulva* relative to un-enriched *Ulva* are also shown.

| | Units | Control mean | se | Enriched mean | se | *P*-value | Proportional change |
|---|---|---|---|---|---|---|---|
| **Total inorganic N (seawater)** | µM | 2.27 | 0.17 | 12.52 | 0.45 | **<0.001** | 5.5 |
| ***Ulva* nitrogen index** | | | | | | | |
| total free amino acids | µmol N · g $DW^{-1}$ | 57.8 | 15.0 | 255.2 | 9.2 | **<0.000** | 4.4 |
| asparagine | µmol N · g $DW^{-1}$ | 25.4 | 11.0 | 172.5 | 11.0 | **<0.001** | 6.8 |
| apspartate | µmol N · g $DW^{-1}$ | 0.84 | 0.05 | 2.05 | 0.14 | **<0.001** | 2.4 |
| asparagine : aspartate | | 27.5 | 11.1 | 87.1 | 8.0 | **<0.001** | 3.2 |
| glutamine | µmol N · g $DW^{-1}$ | 3.27 | 0.75 | 6.27 | 1.10 | **0.041** | 1.9 |
| glutamate | µmol N · g $DW^{-1}$ | 3.44 | 0.34 | 8.43 | 1.04 | **<0.001** | 2.5 |
| glutamine : glutamate | | 0.54 | 0.14 | 0.37 | 0.06 | 0.255 | 0.7 |
| histidine | µmol N · g $DW^{-1}$ | 13.2 | 3.6 | 34.9 | 4.3 | **0.002** | 2.6 |
| proline | µmol N · g $DW^{-1}$ | 1.96 | 0.27 | 8.09 | 1.01 | **<0.001** | 4.1 |
| $U2^{9.48}$ | µmol N · g $DW^{-1}$ | 0.37 | 0.05 | 8.13 | 1.77 | **<0.001** | 22.1 |
| other amino acids | µmol N · g $DW^{-1}$ | 24.8 | 5.3 | 65.9 | 4.9 | **0.038** | 2.7 |
| chlorophyll *a* | µg · g $DW^{-1}$ | 2.50 | 0.58 | 5.48 | 0.15 | **<0.001** | 2.2 |
| chlorophyll *b* | µg · g $DW^{-1}$ | 2.56 | 0.69 | 5.85 | 0.68 | **0.003** | 2.3 |
| chlorophyll *a* + *b* | µg · g $DW^{-1}$ | 5.06 | 1.26 | 11.34 | 0.74 | **<0.001** | 2.2 |
| free amino acid-N : total tissue-N | % | 5.04 | 0.51 | 11.33 | 0.69 | **<0.001** | 2.2 |
| total tissue-N | % | 1.48 | 0.31 | 3.17 | 0.14 | **<0.001** | 2.1 |
| ammonium assimilation | µmol · g $DW^{-1}$ · $h^{-1}$ | 60.8 | 19.5 | 66.7 | 21.8 | 0.864 | 1.1 |
| growth | $day^{-1}$ | 0.10 | 0.01 | 0.15 | 0.03 | 0.210 | 1.5 |

Neither the rate of ammonium assimilation nor the rate of growth provided a significant reflection of increases in seawater nitrogen availability.

### *Nitrogen status, and light and season*

While nitrogen content and other N-indices (e.g., chlorophyll) in *Ulva* were affected by nitrogen concentration in seawater (and water motion, Figure 3.13), these parameters were also affected by differences in light and season. From combined data the figure below (Figure 3.19) shows three important features: 1) Enriched *Ulva* grown in the winter tended to have the highest values of TN and chlorophyll overall, 2) There was one example of unenriched *Ulva* in winter that contained as much nitrogen and chlorophyll as enriched *Ulva* in the summer, and 3) both chlorophyll *a* and, to a lesser extent *b*, were well correlated with TN (Figure 3.19).

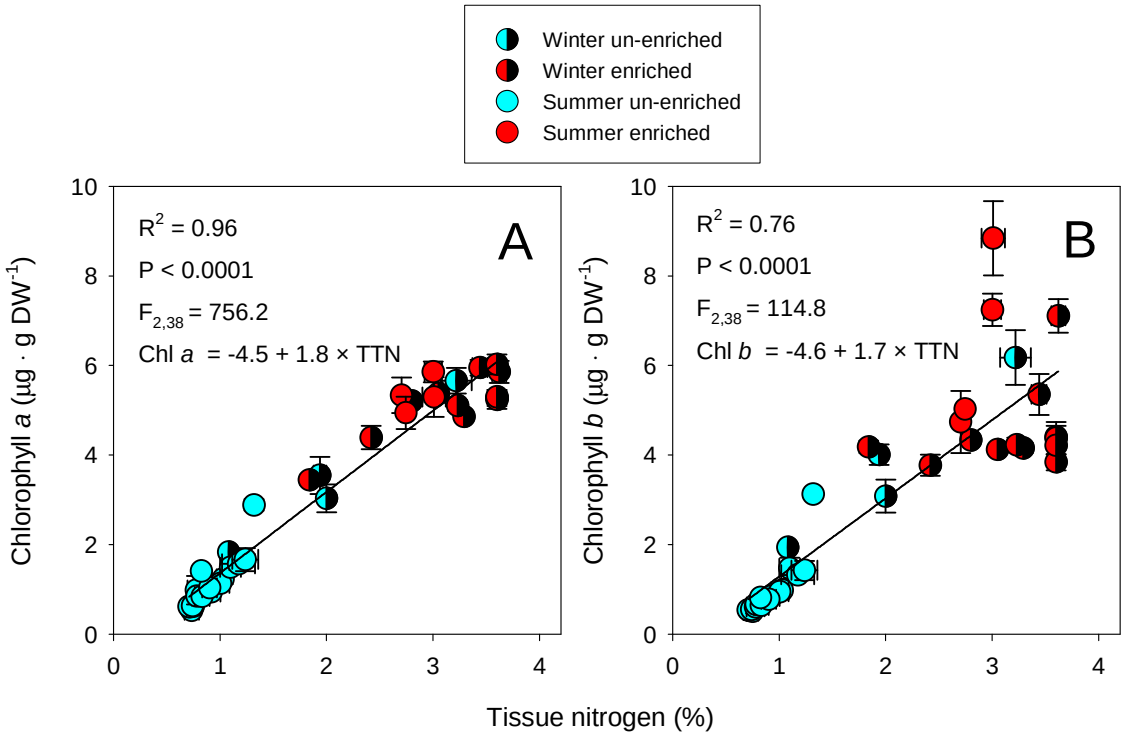



**Figure 3.19.** Combined results of change in chlorophyll (**A**) *a* and (**B**) *b* with change in total tissue nitrogen content in *Ulva pertusa*.

In addition to differences in *Ulva* nitrogen status between seasons, and between light treatments, there was also apparent effects on the alga's growth rate. Both Experiments 1 and 4 showed that growth rate in *Ulva* was higher in unshaded conditions compared with shaded conditions (Figure 3.3 and 3.14), irrespective of season. However, there was also some suggestion that growth may also have been limited in unshaded controls by nitrogen or phosphorus supply in the summer (Figure 3.14, Experiment 4). Combined data from all experiments suggests that overall growth rates in *Ulva* were higher in summer compared with those measured in the winter, although *Ulva* containing lower (around 1 %) TN levels in the summer also had lower growth rates. Moreover, there appeared to be a hyperbolic relationship between growth rate and TN content (Figure 3.20). In contrast to growth rate, nitrogen content tended to be higher in *Ulva* grown both under winter conditions (compared with summer) and also, to some extent, under shaded (compared with unshaded) conditions (Figure 3.20). Moreover, the lowest growth rates and some of the highest values of TN content co-occurred in *Ulva* grown in the winter under

shaded conditions (Figure 3.19). On the other hand the highest growth rates occurred in *Ulva* that only had low to intermediate values TN content (Figure 3.20).

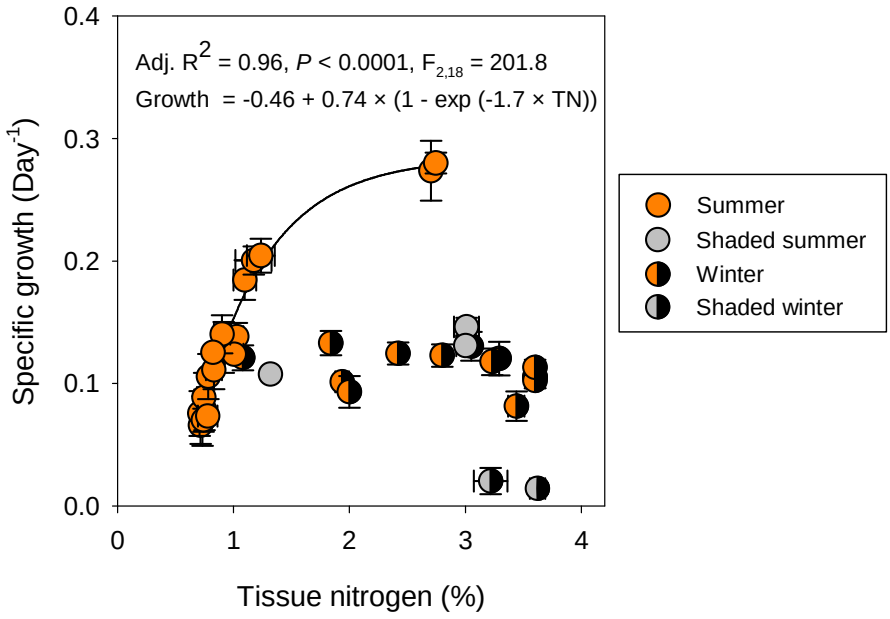



**Figure 3. 20.** Combined results of changes in growth rate with change in total tissue nitrogen content in *Ulva pertusa*. Note that a exponential rise to maximum curve is fitted to unshaded summer data only.

Maximum rates of ammonium assimilation and levels of glutamine (a key amino acid involved in ammonium assimilation, see Chapter One) were also examined in the context of light and season. From combined results of Experiments 1 and 4 (note that rates of ammonium assimilation were only measured in these two experiments) there were both lower rates of ammonium assimilation and lower levels of glutamine in *Ulva* grown under both shaded (probably light-limited) conditions and in winter (Figure 3.21). Conversely, higher values of both ammonium assimilation and glutamine were found in *Ulva* grown in summer (Figure 3.21).

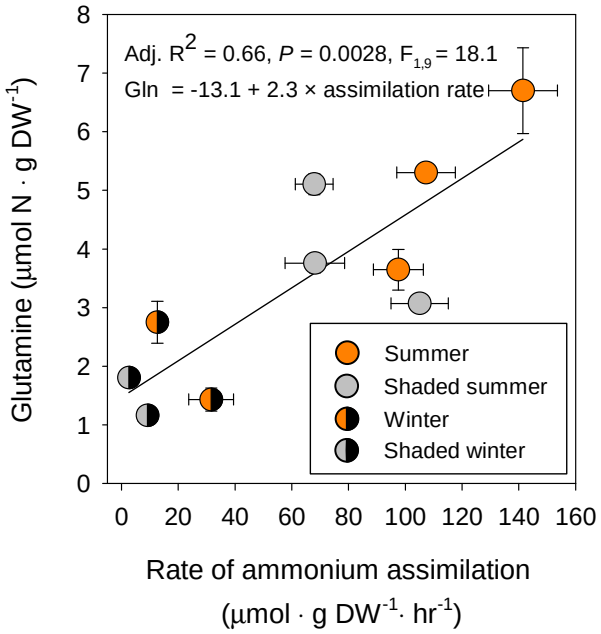



**Figure 3.21.** Combined results from summer and winter enrichment experiments (Experiment 1 and 4) of changes in glutamine content with change in the maximum rate of ammonium assimimilation in *Ulva pertusa*.

***Nitrogen isotopes, light and season***

All un-enriched controls used in experiments in this chapter showed a narrow range of values between 6.8 ± 0.1 and 8.1 ± 0.1 ‰, and showed little or no seasonal shift in values compared with values seen in *Ulva* supplied with synthetic nitrogen sources (Figure 3.22). It was noted that this range was very similar to the range seen in natural populations of *Ulva* growing at exposed and presumably minimally impacted rural sites examined in summer and winter in Chapter Two (Figures 2.5 and 2.6). In contrast to the narrow range seen in *Ulva* supplied with natural seawater, the addition of synthetic nitrogen sources (either as ammonium chloride [$NH_4Cl$] or sodium nitrate [$NaNO_3$]) substantially altered the $\delta^{15}N$ signature seen in *Ulva* tissues. In addition, it was apparent that $\delta^{15}N$ values in ammonium enriched *Ulva* tended to increase with increasing TN content (Figure 3.22). $\delta^{15}N$ values in nitrate enriched *Ulva* were more intermediate between *Ulva* supplied with natural seawater and that supplied with ammonium enriched seawater.

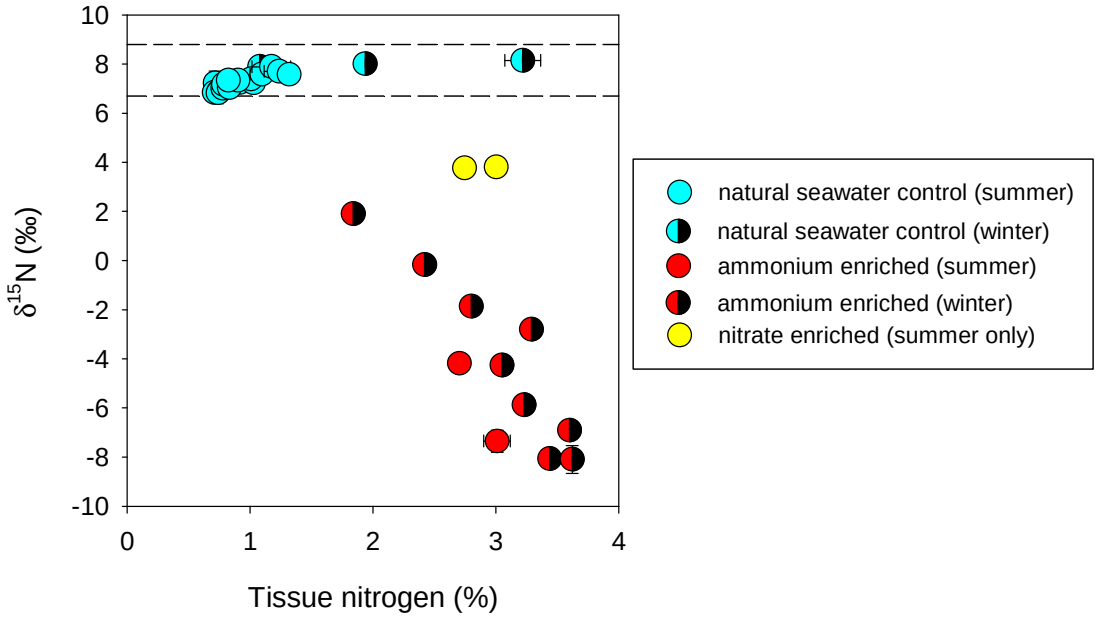



**Figure 3.22.** Combined results of change in tissue $\delta^{15}N$ values with change in total tissue nitrogen content in *Ulva pertusa*. The dashed lines represent the range of values found in *Ulva* growing on rural exposed coasts around New Zealand (Chapter Two).

## 3.5 Discussion

***Nitrogen indices, water motion and seawater nitrogen concentration***

The main purpose of this chapter was to identify N-indices that were most likely to reflect differences in seawater nitrogen concentration and ideally be robust against other factors such as season or light. Contrasting differences in nitrogen status of *Ulva* was usually visually apparent in the 'greenness' of the thalli. However, the clearest measured biochemical response to nitrogen addition in seawater was the FAA pool. Although the FAA pool began to saturate at concentrations in seawater above about 10 µM (Figure 3.9, A), overall it exhibited a far greater (21-fold) range of values (16 µmol N · g $DW^{-1}$ to 347 µmol N · g $DW^{-1}$) than either chlorophyll or TN, suggesting that it could be better at resolving differences in available nitrogen in seawater. On the other hand, there was some evidence (e.g., in Experiment 1, Figure 3.5, A) that the total FAA pool continued to change over long (1 month) time periods and was probably affected to some extent (along with other N-indices) by factors other than external nitrogen concentration. Another important factor (although not

examined here in the context of nitrogen addition) was that of water flow rate and water motion.

Flow rate and water motion not only affected the FAA pool in *Ulva* but also TN and chlorophyll content, and growth. Quantitatively though, the addition of nitrogen appeared to have a much greater affect on the FAA pool compared with water motion alone (i.e., the highest FAA concentration attained with the highest level of turbulence used in Experiment 3 was 45.4 ± 16.9 μmol N · g $DW^{-1}$ compared with 347.3 ± 16.6 μmol N · g $DW^{-1}$ achieved with 20 μM nitrogen addition and turbulence). This might be expected since high nutrient concentrations in theory should be more dominant over water motion in minimising the effect of diffusive boundary layers in *Ulva* (Parker, 1981). In addition, the linear relationships that were suggested between FAA and flow rate (with and without turbulence, see Figure 3.13), in comparison with the clear nonlinear responses of the TN and TChl (and growth), might suggest that changes in the FAA pool are more closely linked to fine scale changes in the flux of nutrients past the thallus surface (i.e., small changes in nitrogen flux are probably equivalent to very small changes in nitrogen concentration). However, results from a preliminary experiment also suggested that even at moderately low (about 10 cm · $min^{-1}$) flow rates, with moderate (10 μM) nitrogen addition, there was little or no effect of turbulence on the FAA pool in *Ulva* (data not shown). In other words the effect of turbulence on the accumulation of FAA pool in *Ulva* was probably dominated by the effect of high nitrogen concentration.

***Free amino acids as indices of seawater nitrogen concentration***

Individual free amino acids in macroalgae have previously been used to indicate nitrogen availability in seawater (e.g., Horrocks *et al.*, 1995; Jones *et al.*, 1996; Costanzo *et al.*, 2000; Barr and Rees, 2003). Except for the study of Barr and Rees (2003) which examined short-term changes in amino acid and tissue ammonium content in *Ulva intestinalis*, the other studies listed above used *Gracilaria* sp. The study of Jones (1996) showed that some amino acids in *Gracilaria edulis* responded differently to different nitrogen sources (i.e., ammonium-enrichment produced increases in citrulline, phenylalanine and serine while nitrate-enrichment produced increases in glutamate, citrulline and alanine). In the current study the strongest differential response of any amino acid to nitrogen source (nitrate versus ammonium)

was in higher levels of proline (see Figure 3.16, F) in *Ulva* supplied with nitrate in unshaded conditions. Otherwise there was little evidence to suggest that free amino acids in *Ulva* could reliably distinguish between the two nitrogen sources.

In the study of Taylor *et al* (2006) which examined short-term (up to 10 hr) responses of amino acids in *Ulva intestinalis* to nitrate and ammonium addition, both glutamine and asparagine increased more with ammonium than with nitrate addition. However, in the current study after 12 days in constant, relatively low (10 μM) concentrations, there was little difference in the size of the FAA pool in *Ulva pertusa* supplied with equivalent concentrations of ammonium and nitrate (Figure 3.15, C). Given that the FAA pool in nitrate-supplied *Ulva* was initially smaller, and that growth rates were initially lower than in ammonium-supplied *Ulva*, it is concluded that it took time for *Ulva* to acclimate to nitrate utilisation. What was clear from the current study was that light-limited conditions affected levels of both glutamine and glutamate (Figure 3.16, C and E). This was probably the result of a reduction in levels of carbon skeletons (probably in the form of 2-oxaglutarate) required for ammonium assimilation via GS-GOGAT. Additional evidence for the effect of light on nitrogen assimilation comes from the observation that there were lower (maximum) rates of ammonium assimilation and lower levels of glutamine in *Ulva* grown in both light-limited conditions and in winter (Figure 3.21).

Glutamine levels in *U. pertusa* constantly supplied with 10 μM ammonium made only a small (2.1 %) contribution to the FAA pool compared with the large (up to 60.1 %) contribution of glutamine observed in *U. intestinalis* after either short-term ammonium-enrichment experiments (Taylor *et al.*, 2006) (Table 3.7) or in natural intertidal populations after tidal re-supply of nitrogen (Barr and Rees, 2003). Conversely, asparagine levels in *Ulva*, given a constant nitrogen supply, represented a much greater (69.6 %) contribution to the FAA pool compared with the 20 % contribution obtained in short-term experiments (Table 3.7). In addition, after time the total FAA pool was much larger in *Ulva* supplied with 10 μM ammonium compared to levels in *Ulva* under short-term enrichment at much higher (400 μM) ammonium concentrations (Table 3.7). Irrespective of the differences between short and long-term responses of *Ulva* to nitrogen supply, accumulation of asparagine strongly suggests that this amino acid plays an important storage role and this is

consistent with what is known about nitrogen metabolism in higher plants. Moreover, the ultimate dominance of asparagine over glutamine possibly relates to it being more soluble and less reactive than glutamine, therefore making it a more effective N storage molecule in higher plants (Sieciechowicz *et al.*, 1988) and apparently in *Ulva*.

Compared with the study of Taylor *et al* (2006) and Barr and Rees (2003) glutamine remained at fairly low levels averaging about 4.1 ± 0.4 μmol N · g $DW^{-1}$ for all experiments. This was also true for some *Ulva* samples collected from around New Zealand (Chapter Two) where glutamine levels, even from some enriched sites, were often low. In addition, it was apparent from Experiment 2 that glutamine and glutamate responded differently (Figure 3.11, A and B) to changes in nitrogen concentration and might reflect some form of metabolic regulation, possibly down-regulation, of nitrogen assimilation. It is known that the level of glutamine and / or the ratio of glutamine : glutamate is an indicator of N-status in bacteria (Ikeda *et al.*, 1996), microalgae (Flynn, 1990b) and higher plants (Watanabe *et al.*, 1997). However, there are no studies that specifically examine regulation of nitrogen assimilation in subtidal macroalgae in response to increasing nitrogen concentration. From the current study glutamate, the net product of GS-GOGAT, was present at lower levels at higher (> 10 μM) TIN concentrations suggesting that either glutamate was increasingly being incorporated into some other unmeasured storage pool or

**Table 3.7.** Comparison of changes in free amino acid content in *Ulva intestinalis* in short-term ammonium enrichment (400 μM) experiments (Taylor *et al*, 2006) with combined results for *Ulva pertusa* maintained long-term under low (10 - 12 μM) and constant concencentrations of inorganic nitrogen. Note that only unshaded, ammonium-enriched treatments were used to standardise the comparison with Taylor *et al,* 2006.

| | Short-term ammonium (400 μM) enrichment (From Taylor *et al,* 2006) | | Long-term ammonium (10 μM) enrichment (Current study) | |
|---|---|---|---|---|
| | umol N $gDW^{-1}$ | % total | umol N $gDW^{-1}$ | % total |
| Aspartate | 1.5 ± 0.1 | 1.8 | 2.0 ± 0.2 | 0.8 |
| Asparagine | 13.7 ± 0.8 | 16.1 | 178.9 ± 13.9 | 69.6 |
| Glutamate | 3.6 ± 0.1 | 4.3 | 8.0 ± 1.3 | 3.1 |
| Gluamine | 51.7 ± 2.5 | 60.7 | 5.4 ± 1.3 | 2.1 |
| Others | 14.6 ± 1.0 | 17.2 | 62.8 ± 5.5 | 24.4 |
| Total | 85.2 ± 4.6 | 100 | 257.0 ± 11.7 | 100 |

decreased because of a reduction in GOGAT (2-oxoglutarate aminotransferase) activity (as opposed to that of GS (glutamine synthetase)). It is interesting to note that the responses seen here have also been shown in higher plants. In experiments on tobacco leaves Geiger *et al* (1999) showed that levels of glutamate decreased with high, but not intermediate, nitrogen concentrations while glutamine showed a proportional increase with increases in nitrogen supply.

Most (14 out of 17, Table 3.3) of the amino acids examined in this study correlated with the total FAA pool suggesting that they were also either directly or indirectly affected by changes in external nitrogen supply. In addition to increases in asparagine most other amino acids (except glutamate) also showed increases with nitrogen concentration. The most predominant of these included proline, histidine and the unknown amino compound $U2^{9.48}$. Increases in proline occur in response to environmental stress, such as extremes of temperature or changes in salinity in both higher plants (Matysik *et al.*, 2002) and algae (Ahmad and Hellebust, 1988b; Singh *et al.*, 2005; Kakinuma *et al.*, 2006).

Another amino acid found in higher concentrations was histidine. Although it does not have a direct role in nitrogen metabolism, it is involved in chelating and transporting metal ions in plants, and with 3 N atoms it may confer a nitrogen storage role. In addition to the amino acids mentioned above, most of the minor free amino acids in *Ulva* contribute significantly (24 %) (Table 3.7) to the nitrogen stored in the FAA pool. This same tendency has been shown in higher plants (Ireland, 1998) and microalgae (Flynn, 1990a) and it appears that regardless of the form of nitrogen supplied (nitrate or ammonium), there is subsequent accumulation of most amino acids, presumably distributed in different subcellular compartments. To the best of my knowledge this is the first study on macroalgae which demonstrates that the pool of amino acids and most of its constituents are strongly influenced by nitrogen supply and therefore, in addition to their individual metabolic roles, probably also have a nitrogen storage function in *Ulva*.

One notable feature of all the tissue N indices examined (FAA, chlorophyll and TN) was that they showed a very clear saturating response to nitrogen concentration. However, in the study of Jones (1996) linear relationships between levels of some

amino acids and seawater nitrogen concentrations were reported for seawater nitrogen concentrations up to 800 μM, although these were only from relatively short (3 day) incubations. The linear responses of amino acids reported for *Gracilaria* probably relate to the fact that these were short-term experiments and were not likely to be at equilibrium with external nitrogen concentration. It is suggested that in the long term changes in *Gracilaria* may have resulted in larger changes (relative to non-enriched conditions) in all N pools, including that of the FAA pool; neither levels of total FAA or glutamine were reported in this study.

In an indoor prototype of the system used in the current study, nitrogen enrichment of two species of *Gracilaria* using the same concentrations used in Experiment 2 (i.e., ambient seawater and ambient seawater plus 10, 20 and 40 μM ammonium), resulted in a similar saturating response to *Ulva* (Wilcox *et al.*, 2006). It is also interesting to note that after two weeks maintained at these concentrations the amino acid pool, dominated by gigartinine, represented up to 47 % of the total tissue nitrogen content in this species. However, these two species of *Gracilaria* showed a much lower range (2.2-fold to 2.9-fold) of total FAA content between non-enriched and the highest concentration (40 μM) compared with that (12-fold) in *Ulva* (see results Experiment 2, 1$^{st}$ paragraph).

***Growth, N-indices and season***

Experiment 4 (conducted in the summer) achieved maximum growth rates in unshaded, ammonium-enriched *Ulva* that were over four times higher than those achieved in unshaded *Ulva* in the winter in Experiment 1 (i.e., 0.08 · day$^{-1}$ and 0.34 · day$^{-1}$ for winter and summer, respectively). Other than seasonal differences in light, temperature and ambient seawater nutrient concentrations, controlled conditions in this experiment (e.g., flow rate and ammonium concentration) were comparable to those of Experiment 1. In greater contrast, shaded treatments (with an 82 % decrease in light) in the summer had much higher growth rates compared with their winter enriched counterparts (i.e., 0.20 · day$^{-1}$ and 0.014 · day$^{-1}$ for summer and winter, respectively). Therefore, the effects of season and light would appear to have had the greatest influence on differences in growth rate (Figure 3.20). However, since only light was examined, the role of temperature cannot be ruled out and is known to be an

important factor affecting growth in algae (Lapointe, 1982; Raven and Geider, 1988; Duke *et al.*, 1989a; Henley and Ramus, 1989b; de Casabianca *et al.*, 2002).

Clearly, nitrogen status alone had no ability to predict growth rate unless examined in the context of seasonal effects (e.g. Figure 3.20). In the summer, growth of *Ulva* was hyperbolically related to nitrogen content (as shown by others e.g., Bjornsater and Wheeler, 1990) suggesting that nitrogen availability was possibly linked to the enhanced growth seen in the summer compared with that in the winter (Figure 3.20). In addition, there was some evidence that *Ulva* grown in the summer and supplied with nitrate alone had a lower growth rate, at least temporarily, than that supplied with ammonium alone (see Figure 3.14). It would appear that change in *Ulva* nitrogen metabolism and growth was more an acclimation response to the sole nitrogen source being nitrate rather than a sustained cost expressed as reduced growth. It is also worth noting that a similar experiment conducted at a later time failed to yield a consistent and significant effect of nitrogen source on the growth rate in *Ulva* (data not shown). It would appear that, in the case of *Ulva* anyway, there is no evidence supporting the hypothesis that nitrate utilisation for growth might come at an energetic cost relative to ammonium (Syrett, 1981; Turpin *et al.*, 1991; Vergara *et al.*, 1995).

Higher levels of TN and chlorophyll (*a* and *b*) occurred in *Ulva* either in the winter months, or in treatments that had been enriched with nitrogen (Figure 3.19). Moreover, the highest TN values occurred in *Ulva* that was enriched in the winter (i.e., the effects of nitrogen enrichment and season were probably additive) (Figure 3.19). In addition, in Experiment 1 shaded, non-enriched *Ulva* had TN and TChl values that were not significantly different from enriched *Ulva*, but were higher than the unshaded, non-enriched control (Figure 3.6, A and B). This would tend to suggest that the lower growth rates observed in light limited (unenriched) *Ulva* in the winter contributed to their higher nitrogen content, despite the lower rates of ammonium assimilation observed in the winter (compared with those in the summer) (Figure 3.21). Furthermore, although seawater total inorganic nitrogen concentrations generally increase in the winter at Goat Island (Dobson, unpublished data) there was no evidence that ambient inorganic nitrogen concentrations over the duration of these experiments were any higher in the winter compared with the summer (2.1 μM and

2.2 μM for winter and summer, respectively). Therefore it is suggested that the higher nitrogen content of *Ulva* (enriched and non-enriched alike) in winter was largely due to accumulation (albeit probably slowly) of nitrogen that was in excess of growth requirements. This tends to support the observations from the previous chapter in which natural *Ulva* populations showed strong seasonal differences with higher nitrogen content in the winter compared with summer.

***Tissue $\delta^{15}N$ isotopes in* Ulva**

The strongest indicator of chemical differences in nitrogen source was that shown by natural abundance stable nitrogen isotopes, $\delta^{15}N$. Obviously this was largely due to $\delta^{15}N$ signatures of synthetic ammonium (-5.50 ‰) and nitrate (3.95 ‰) that contrasted to the background (i.e., natural) source pool in seawater which were presumably around 7 ‰ to 8 ‰. All un-enriched controls in this chapter exhibited a narrow range of values between 6.8 ± 0.1 to 8.1 ± 0.1 ‰, and compared with the changes in TN content, showed little seasonal shift in values (Figure 3.22). This range was very similar to that (6.7 ± 0.1 to 8.8 ± 0.1 ‰) observed in *Ulva* on exposed coastlines from around New Zealand (Chapter Two). It is also consistent with that identified as 'acceptable' for *Ulva* growing in non-sewage impacted coastal waters in a pre / post-sewerage discharge closure study at Moa Point, Wellington (Rogers, 2003).

Studies of different microalgae have shown that $^{15}N$ fractionation in nitrate supplied cultures can range from 2.2 ‰ to 6.2 ‰ (Needoba *et al.*, 2003) and in diatoms may be considerable with values as high as 20 ‰ for ammonium and 5 ‰ for nitrate supplied cultures (Waser *et al.*, 1998). Compared to these studies, overall levels of $^{15}N$ fractionation in *Ulva* (under either light-limited or light-saturated conditions) appear to be relatively small (ranging from 1.3 ‰ to -1.9 ‰) (Figure 3.18, B). It should also be noted that values of fractionation obtained in Experiment 4 were similar to a second experiment (carried out in the same apparatus) which removed, or at least markedly reduced, the effect of the background source pool using an algal (*Ulva*) scrubber situated before the point where synthetic nutrients were added. It is interesting that the total range of fractionation observed in this experiment (in absolute terms 3.2 ‰) was greater than the range (2.1 ‰; being the difference

between 6.7 and 8.8 ‰ above) seen in *Ulva* growing on exposed coasts around New Zealand (Chapter Two) for both summer and winter.

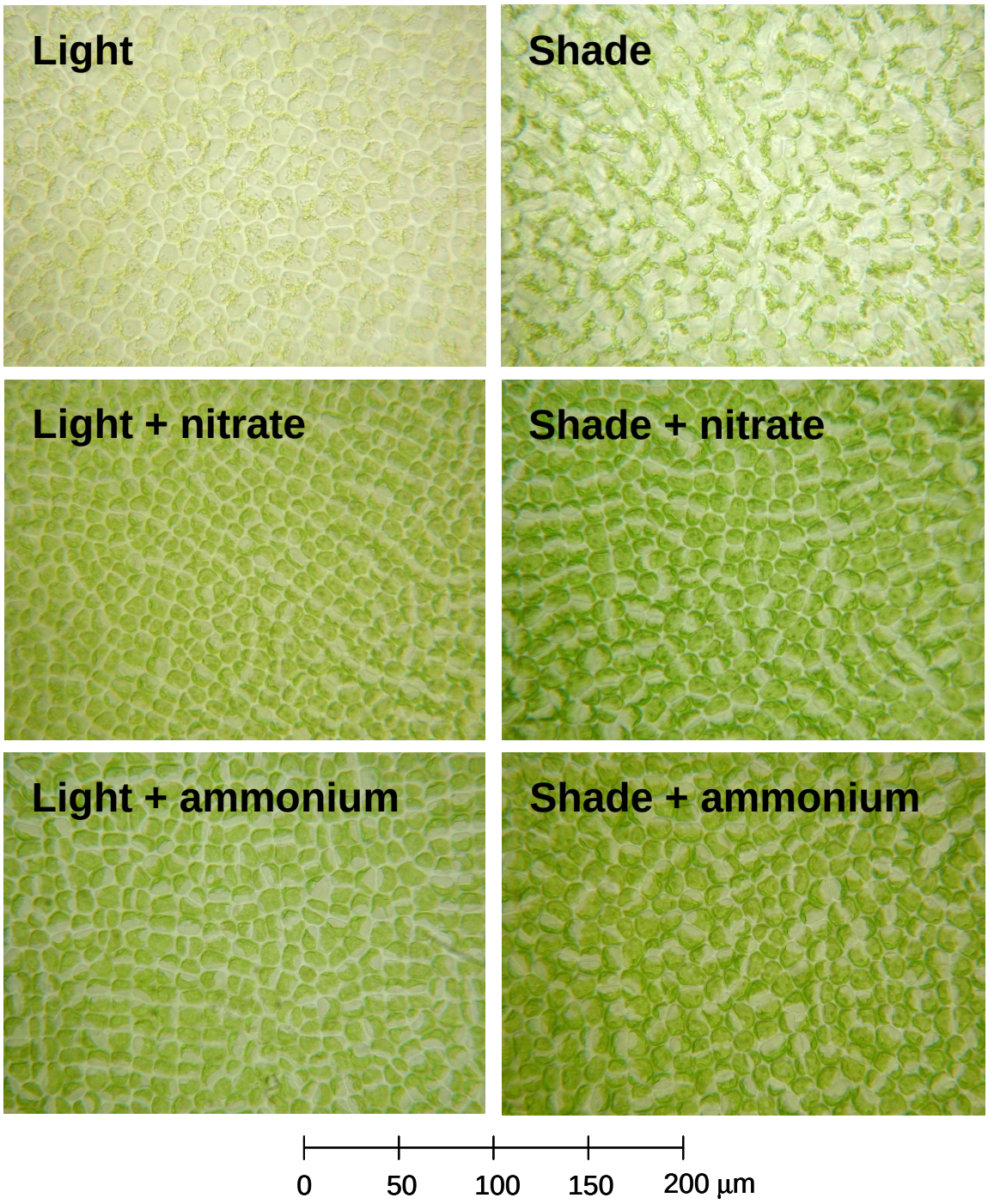
In this experiment the lowest levels of fractionation were seen in *Ulva* supplied with nitrate alone under both light-limited and light-saturated conditions. Given that the free amino acid pool in nitrate-supplied *Ulva* by the end of Experiment 4 (Figure 3.15, C) was similar to that of ammonium-supplied *Ulva* it is presumed that similar amounts of nitrogen from the two nitrogen sources were assimilated. As a corollary it appears that there was little overall effect on net fractionation by nitrate reductase (because of the observed low fractionation values) in nitrate-supplied *Ulva*, irrespective of contrasting effects of light. That ammonium-supplied *Ulva* showed a greater tendency to fractionate between light-saturated and light-limited conditions compared with nitrate supplied *Ulva* is interesting. Fractionation in light-saturated *Ulva* was more positive indicating that there was a either a discrimination against $^{14}N$ at uptake or that there was a loss of $^{14}N$ subsequent to uptake. The former situation seems very unlikely since it is generally accepted that discrimination during biological or physical uptake processes occurs against the heavier $^{15}N$ isotope (Bedard-Haughn *et al.*, 2003). It is possible that in ammonium supplied *Ulva* the effect of light-saturation may have resulted in small changes in cellular pH and therefore the relative proportion of $NH_3$ and $NH_4^+$. In theory in light-saturated *Ulva* a slight increase in pH could have led to efflux of $NH_3$ which in turn might favour the retention of $^{15}N$ resulting in the higher fractionation observed in this treatment. Conversely, under light-limited conditions active transport of $NH_4^+$ may have lead to discrimination of $^{15}N$ at uptake. Why virtually no fractionation of the nitrate N source occurred is unknown, given the greater energetic requirement for utilisation of this source. As noted above it is interesting that Waser (1998) showed that there was more fractionation in ammonium supplied diatoms than those supplied with nitrate.

There appear to be good opportunities for research regarding the actual mechanisms of fractionation in macroalgae. However, what is clear is that *Ulva* closely reflects changes in the source pool apparently with little seasonal effect (Figure 3.22). Although there is very little research with macroalgae in this area it would seem that these conclusions for *Ulva* are supported. For example the work of Cohen and Fong (2005) which examined short-term (hours) changes in *Enteromorpha* (syn. *Ulva*)

*intestinalis* indicated that there was no discrimination of $^{15}N$ in tissue supplied with ammonium and nitrate, and that tissue $\delta^{15}N$ values were independent of N concentration. However, it may be that the slight differences in fractionation observed in the current study were only discernable because tissue turnover occurring through time (14 days) and is more likely to reflect the totality of biochemical changes affecting net fractionation.

***Conclusions***

From the results presented here water motion, season and light were clearly factors that affected growth and N-indices in *Ulva*. However, N-indices in *Ulva* also showed a saturating response to low, but ecologically realistic nitrogen concentrations, and taking at least the aforementioned factors into account, it appears that higher levels of tissue nitrogen, chlorophyll and particularly FAA should indicate the presence of higher (> 10 μM) concentrations of nitrogen in seawater. Moreover, it appears that levels of the FAA pool and probably asparagine are most likely to provide contrasting and less seasonally affected biological measures of nitrogen availability, irrespective of nitrogen source. The results from this Chapter would appear to be consistent with the patterns observed in natural populations, especially those that exist in nitrogen-enriched environments. In conclusion, it is suggested that in combination with *Ulva* tissue $\delta^{15}N$ values the FAA pool, as a quantitative biochemical measure of nitrogen availability, is likely to provide useful information about both the amount and composition of nitrogen entering coastal environments.




**Appendix 3.1.** Comparison of chloroplasts in *Ulva pertusa* maintained under either ambient or shaded light, with and without nitrate or ammonium addition. The Individual plates shown above are representative examples of the six treatments.

# Chapter Four

# Developing *Ulva* as a multi-purpose environmental test-organism

## 4.1 Abstract

Artificial populations of *Ulva* were developed for use as a standardised indicator of seawater quality, with a particular focus on nitrogen loading. To develop *Ulva* as a standardised test-organism it was cultured in low nutrient (non-polluted) seawater to deplete internal storage pools of nitrogen. Each month the resulting test-*Ulva* was then placed in surface moored growth enclosures at a range of coastal sites around Auckland and then monitored for one year. Sites included two low-nitrogen reference sites, a site on the outskirts of metropolitan Auckland and seven urban sites in two large harbours adjacent to metropolitan Auckland. Seawater nutrient concentrations and biochemical nitrogen indices in test-*Ulva* were sampled each month over the course of one year.

Three N-indices were examined for test-*Ulva*: total tissue nitrogen (TN), total chlorophyll (TChl) and total free amino acids (FAA) (and individual amino acids). In winter there were increases in seawater inorganic nitrogen and concomitant increases in FAA content. However, TN and TChl content in test-*Ulva* showed similar increases (possibly saturating) across all sites suggesting that seasonal increases in these N-indices were also due to other seasonal factors (e.g., surface irradiance and / or seawater temperature). On the other hand, total FAA pool and the individual amino acids; glutamine, proline and one unknown amino acid showed strong differences between a low-nitrogen reference site and the other study sites all year round. In addition, it was probable that test-*Ulva* were integrating differences in tidally-averaged nitrogen loading that were not reliably detected in instantaneous seawater samples. Controlled enrichment experiments supported results from field deployed test-*Ulva*.

The ratios of abundant stable nitrogen isotopes ($\delta^{15}N$) were also measured in test-*Ulva* tissues and it was shown that *Ulva* grown in urban environments were consistently characterised by values outside the range of values previously identified in *Ulva* growing in exposed clean seawater sites from around New Zealand. The highest value obtained in the survey (14.7 ‰) was obtained at a site near to a large sewage wastewater discharge. In addition to detecting differences in isotopic composition and levels of nitrogen, test-*Ulva* from different environments also accumulated different metals at different concentrations. It is likely that most of the metals accumulated in *Ulva* tissues were biologically-available from the dissolved fraction in seawater. In the more sediment impacted sites Al and Fe accumulated at tissue concentrations close to two orders of magnitude greater than the other metals present. It was therefore concluded that in addition to monitoring nitrogen availability, a variety of other environmental contaminants could potentially be monitored using a single standardised test organism such as *Ulva*.

## 4.2 Introduction

Monitoring changes in the levels of nutrients and metal contaminants in coastal environments has historically been done by direct measurement of water column nutrients. However, instantaneous seawater samples, even when taken regularly, may not give good information on changes in biologically significant nutrient concentrations in environments that are prone to tidal variation in nutrient supply (Björnsäter and Wheeler, 1990; Jones *et al.*, 1996; Valiela *et al.*, 1997; Fong *et al.*, 1998). In addition, conventional seawater analysis does not indicate all sources of nitrogen available to primary producers. Therefore there may be little correlation between water column nitrogen or phosphorus concentrations and either productivity or growth of primary producers (Fong *et al.*, 1998).

Partly as a result of the shortcomings of conventional seawater monitoring techniques several studies have examined the utility of macroalgae as bioindicators of nutrient enrichment (Fong *et al.*, 1994; Jones *et al.*, 1996; Munda and Veber, 1996; Fong *et al.*, 1998; McClelland and Valiela, 1998; Campbell, 1999; Rogers, 1999; Costanzo *et*

*al.*, 2000; Costanzo *et al.*, 2001; Jones *et al.*, 2001; Umezawa *et al.*, 2002; Barr and Rees, 2003; Cohen and Fong, 2004b). In theory there are a number of advantages to using macroalgae as bioindicators of seawater quality, including those advantages involving increased nitrogen loading. Macroalgae are mostly sessile and often perennial, which means they have the ability to accumulate contaminants such as heavy metals, over long periods of time at one site. From the point of view of monitoring levels of nitrogen loading, nitrogen status of macroalgal tissue is generally believed to reflect nitrogen availability in seawater (Duke *et al.*, 1989a; Jones *et al.*, 1996; Fong *et al.*, 1998). In addition, macroalgae, such as *Ulva*, have been shown to take advantage of highly transient pulses in nutrient supply that might be missed by routine seawater sampling (Pedersen, 1994).

Given the characteristics identified above, macroalgae such as *Ulva*, could be viewed as indicators of nitrogen availability because they store nitrogen that is surplus to growth requirements. However, there are potential difficulties with interpreting N-status in natural populations of macroalgae, including species such as *Ulva*, as N-status tends to vary with season (as demonstrated in Chapter Two). Moreover, natural populations of a selected test species may not exist in some of the marine environments we wish to examine. However, it should in theory be possible to overcome these types of limitations if a standardised population of algae is used for the test. In order to achieve this, the test-alga would have to be robust and able to grow in a range of different environments. The opportunistic green alga *Ulva* grows in many environments and displays a high degree of physiological plasticity in response to nitrogen loading and therefore is an ideal candidate for this purpose.

What information is required if macroalgae are to be used as reliable indicators of seawater nitrogen concentrations, assuming that measurements of the latter are spatially and temporally unreliable? There are at least two areas of concern. The first is that the indicator (e.g. amino acid content) should respond to seawater nitrogen concentrations only. In other words, nothing else in the environment should have any effect on the indicator. We currently lack sufficient information on this aspect, and it is possible that any indicator is as unreliable as measurements of seawater nitrogen concentrations. Secondly, it would be extremely difficult to verify independently that the indicator was a valid measure of seawater nutrients in the field. If measurements

of seawater nitrogen concentrations are unreliable, what can the indicator be compared with to determine its reliability? Without such a comparison, it would be, at best, very difficult to determine if the indicator was reliable. This Chapter consequently concentrates on the general relationships between seawater inorganic nitrogen concentrations and the physiological and biochemical responses of deployed *Ulva*. In the process, aspects that may be useful to future attempts to develop biological indicators of seawater nitrogen concentrations are highlighted.

The primary aim of this chapter was to develop a way of using *Ulva* as a biological indicator of seawater quality, particularly nitrogen loading. To achieve this, an integrated system was developed which included:

1) A simple method of routinely maintaining and on-growing a supply stock of non-polluted *Ulva*
2) A system for manipulating these plants to produce a standard, low nitrogen test-*Ulva*
3) A deployment system that was robust, cost-effective and simple to maintain in field conditions.
4) Standard methods for measuring the biochemical responses of test-*Ulva.*
5) Monitoring both seawater nutrient concentrations and the biochemical responses of test-*Ulva* over a prolonged period (1 year)
6) Analysing the correlations between the biological responses of test-*Ulva* and the physical environment.
7) Experimental validation of biochemical responses of test-*Ulva* using laboratory-based manipulative experiments.

## 4.3 Methods

***Laboratory and Field Apparatus***

### 4.3.1 Seaweed on-growing system.

An apparatus was constructed to on-grow and maintain a stock supply of *Ulva*. The algae were maintained in a large black polypropylene bin measuring 2.4 m × 1.2 m × 0.2 m deep, supported on a simple wooden frame. To provide natural turbulence, seawater was delivered via three 20 L plastic dump buckets equipped with 25 mm plastic sleeves (to act as bearings) inserted through a pivot point, approximately in the geometric centre of gravity in the side of the bucket. The three dump buckets were positioned on a 20 mm galvanized pipe located longitudinally over the rear edge of the tank. This pipe acted as a pivot for all three dump buckets which were positioned equidistantly along the length of the tank at the rear. A second pipe was positioned above and behind the first to act as a stop for the buckets when they tipped. The buckets were counterbalanced with lead weights (not in contact with the seawater) such that when the bucket was full it tipped its contents into the tank, then returned to the upright filling position. The vertical distance between the base of buckets and the water's surface was approximately 200 mm (Figure 4.1 and Plate 4.1).

The main tank was arranged on a slight incline (approximately 4 ° on its longitudinal edge) and was lowest to the rear of the apparatus. The purpose of this incline was to use the net movement of seawater (being towards the rear of the apparatus) to transport debris towards a weir at the rear of the tank. This meant that the system was to a large degree self-cleaning. A fence of coarse plastic mesh (20 mm) was positioned at the rear of the tank to prevent any whole portions of *Ulva* from being lost. Low nitrogen (~ 2.4 μM), Goat Island seawater was constantly delivered to each of the three dump buckets at a rate of about 2 $L \cdot min^{-1}$ per bucket. For the duration of the monitoring experiment (December 2002 to February 2004) the mean seawater total inorganic nitrogen concentration was 2.4 ± 0.2 μM. Finally the whole system was covered with horticultural, 25% light reduction shade cloth.

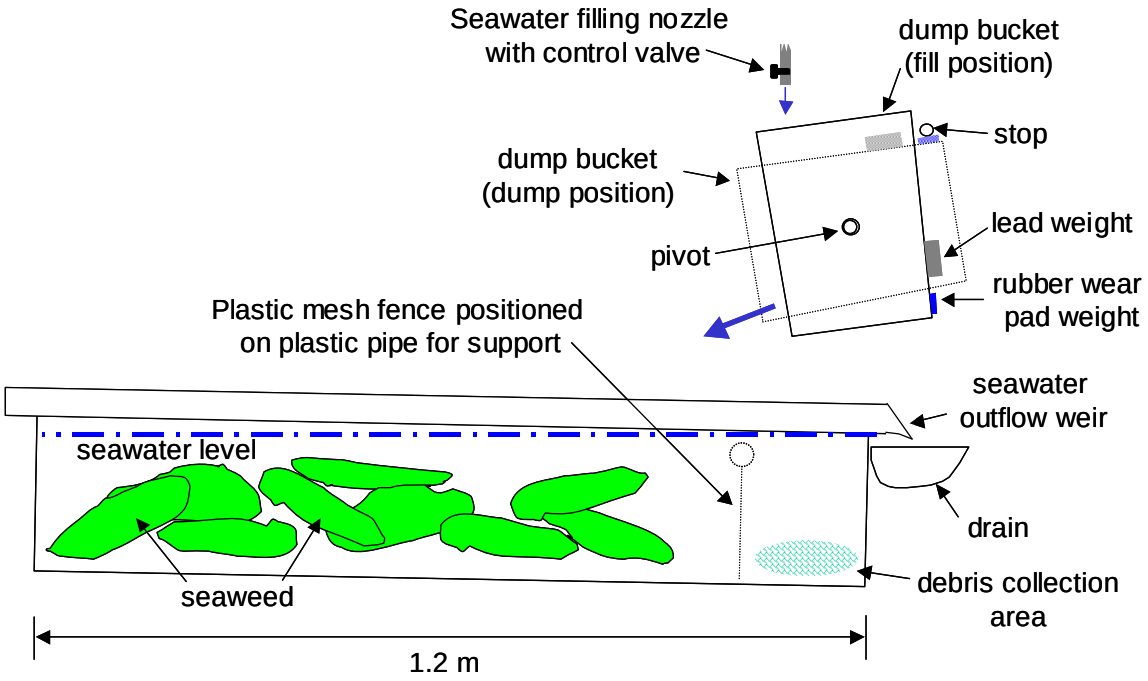



**Figure 4.1.** Turbulent seaweed holding and on-growing tank.

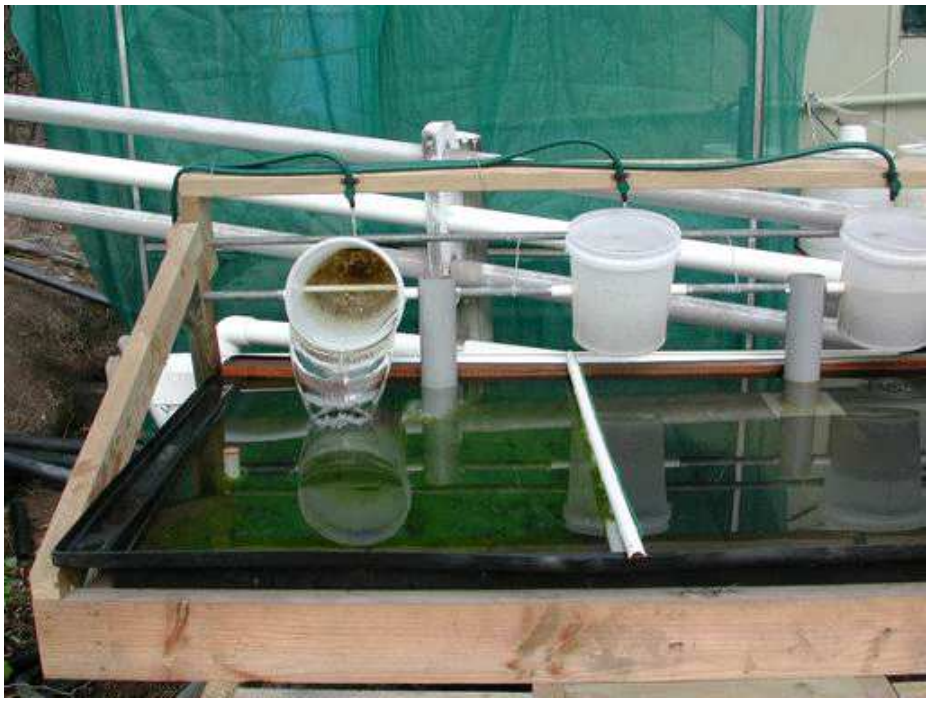


**Plate 4.1.** Turbulent seaweed on-growing system

### 4.3.2 Low nutrient algae growth system.

A second apparatus was constructed to standardise the nitrogen status of *Ulva* prior to field deployment. For the purposes of this study the term "test-*Ulva*" refers to either wild harvested or cultured *Ulva* whose nitrogen status has been decreased under controlled conditions as described below.

Nitrogen drawdown was achieved by maintaining *Ulva* for up to one month in low-nutrient seawater under fluorescent lights (at a photon flux density of 350 $\mu E \cdot m^{-2} \cdot s^{-1}$ [photosynthetically active radiation with a 12:12 h light/dark cycle] using Phillips New Generation TLD 36W / 6500$^{o}$ Kelvin colour temperature fluorescent tubes). Growth chambers were constructed of polypropylene tubs measuring 0.47 m long × 0.18 m wide × 0.13 m high, containing approximately 4 L seawater. The chambers were maintained at a constant temperature of 17.5 ± 1.0 °C. Turbulence was supplied to each chamber using a perspex paddle connected by an eccentric cam to a shaft, which was in turn driven by a windscreen wiper motor (Figure 4.2 and Plate 4.2). Low-nutrient seawater was automatically added (controlled by a time-clock and 24 Vac solenoid valve) via a 80 µm filter for 1 minute every hour. An average daily seawater flow rate was chosen such that the water volume (4 L) of each growth chamber was replaced approximately once per day.

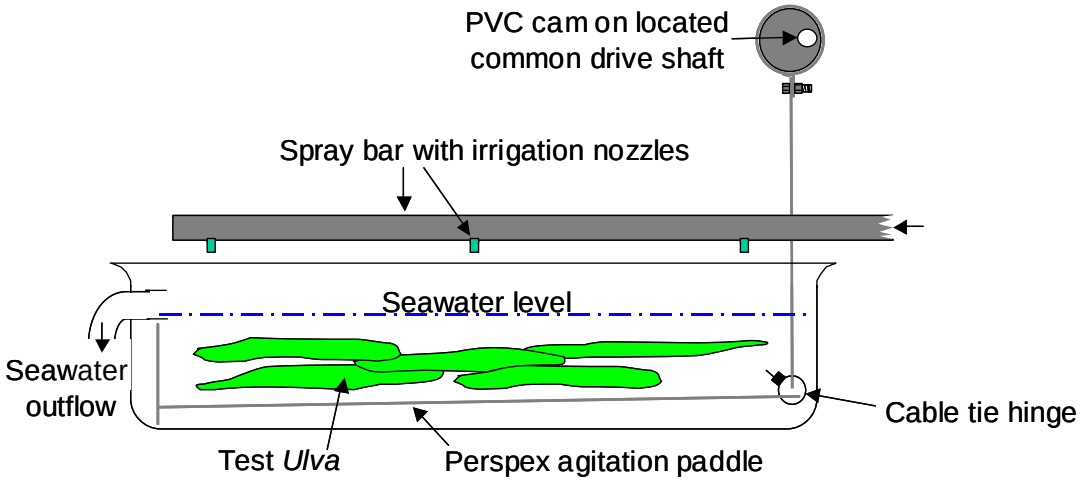



**Figure 4.2.** Schematic of growth chamber used to maintain *Ulva* in artificial light and low nutrient conditions.

**Plate 4.2.** Growth chambers used to maintain *Ulva* in artificial light and low nutrient conditions.

### 4.3.3 Fixed, Artificial Reusable Seaweed Enclosure System (F.A.R.S.E.S.).

A method for maintaining test-*Ulva* in field enclosures was developed. Several prototypes were tested over a period of a year; most of these were not successful. Initially 1 L polyethylene (PET) jars with several 10 mm holes drilled in the ends to allow the flow of seawater through them were used (Plate 4.3, A). A second prototype was developed which involved the use of a simple plastic frame measuring about 150 mm in diameter and constructed out of 5 mm PVC welding rod over which vegetable netting (10 mm mesh) bags were placed (Plate 4.3, B). In the final design net floats were used as an integral part of the frame design (Plate 4.3, C). The use of vegetable netting meant enclosures were easy to clean and maintain since nets were simply replaced at each visit. The open mesh of the netting also meant that seawater could easily flow through the enclosures.

Frames were secured to the mooring lines with a 6 mm rope with quick release clips ('shark clips') to facilitate collection, sample processing and redeployment of enclosures from a boat. Mooring anchors were constructed from short lengths (500 mm) of waratah stakes driven into the soft sediment substrate. This proved to be the cheapest and most reliable method for anchoring the moorings. In cases where the water was too deep to drive stakes a concrete block was used as a mooring anchor. Floats were constructed from recycled 2 L carbonated soft drink bottles with labels containing information about the purpose of the moorings and contact details in case they became dislodged. Three separate replicate moorings were located within about 20m of each other at each site. Over two years (2002 to 2004) 30 of these moorings were used at a variety of sites around Auckland and only five were lost. Spare moorings were always carried during sampling visits.

Initial trials used *Ulva* deployed in subsurface (200 mm below the seawater surface, Figure 4.3, B) chambers, which resulted in heavy fouling of both enclosures and often


**Plate 4.3.** Three prototypes of moored artificial seaweed enclosures used in experiments conducted in Auckland in 2001 to 2003. **A**) Prototype 1, **B**) prototype 2 and **C**) prototype 3. Floats constructed from recycled 2 L carbonated soft drink bottles with labels containing information about the purpose of the moorings and contact details.

*Ulva* tissue. This in turn appeared to affect the health of *Ulva* with much of the tissue either sporulated (non-pigmented) or in an otherwise moribund condition. In subsequent trials using surface moorings (Figure 4.3, A) there was less fouling on growth chambers, particularly on the above-water section (Plate 4.3). In addition, test-*Ulva* showed more vigorous growth and remained in a much healthier condition over the deployment period. It was also reasoned that the biochemical responses of *Ulva* grown in surface moorings were less likely to be confounded by the differential effects of light (e.g., resulting either from fouling or variable levels of turbidity between sites). Note that seawater in several of the sites examined was often turbid (see monitoring site descriptions later). Therefore the use of surface moorings was adopted as standard practice.

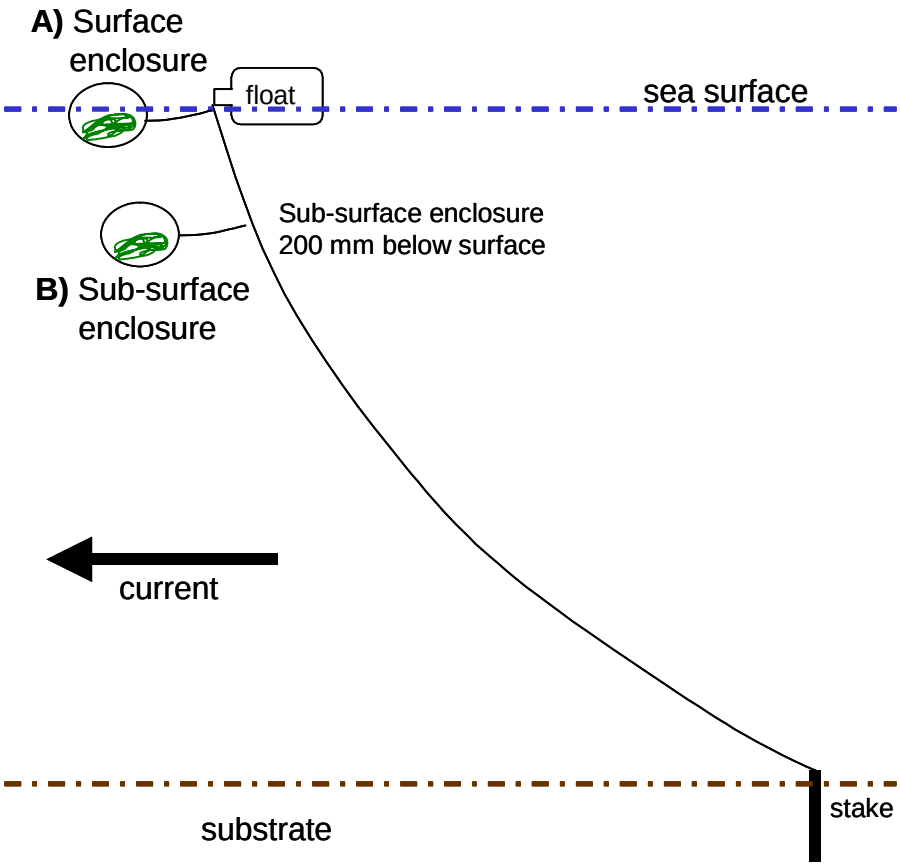



**Figure 4.3.** General layout of moored artificial seaweed enclosures used in experiments conducted in Auckland from 2001 to 2004. (**A**) Surface enclosure and (**B**) sub-surface enclosure located 200 mm below surface.

Finally, it should also be acknowledged that since both *Ulva* and seawater was sampled from the surface, inferences about water quality will be limited to surface waters. However, it is also probably worth noting that since all sites were located in shallow water (2 – 5 m at low tide) in harbours and embayments with large tidal excursions, it is likely that the water columns in these areas were mixed.

### 4.3.4 Artificial outdoor algae nutrient enrichment system

To compare the responses of test-*Ulva* in the field, algae were also maintained under different nitrogen regimes in the apparatus described in Chapter Three.

***Experimental***

### 4.3.5 Test-*Ulva* field trials.

During 2002, trials were conducted using *Ulva* collected from a variety of sites to find a good indicator candidate. Selection criteria included robustness to experimental manipulation and ease of collection from stable, year-round populations from sites with minimal anthropogenic influences on seawater quality. Algae not only had to survive collection from the native site and deployment into low nutrient culture conditions, but also subsequent redeployment into a range of different 'experimental' field environments. After depletion of tissue nitrogen, test-*Ulva* was deployed into the field to determine that its subsequent responses were due to the nutrient status of the new environment rather than that of the recent nutrient pre-history of the *Ulva*.

The most successful of the trials involved the use of *Ulva pertusa* (Maggs and McIvor, 2004) from the Mokohinau Islands (35° 54' 27.29 S, 175° 6' 25.99 E), which being remote was not likely to have been affected by pollution. Trials continued to use *Ulva* from this location for most of 2002, however by the end of 2002 to avoid problems of obtaining a regular supply of wild *Ulva,* self-seeded germlings (possibly derived from the wild Mokohinau Islands population) were collected from growth chambers and maintained in the on-growing system as described in section 4.3.1. This entity proved to be very robust to experimental manipulation and resilient to fouling, and was therefore chosen as the basis of a nutrient monitoring programme in Auckland during 2003 - 2004.

This cultured *Ulva* (and *U. pertusa* from the Mokohinau Islands) had an important distinction from most of the other samples examined in Chapter Two (including *U. pertusa* from Tauranga Harbour as covered in more detail in Chapter Three) in that it didn't accumulate high levels of asparagine when enriched with nitrogen. The reason for this difference is unknown, although it is possible that a genetic factor was involved in the lack of asparagine synthesis in this *Ulva* entity.

### 4.4.6 Experiment 1. Field equilibration of test-*Ulva*.

To evaluate how long it would take to equilibrate to field conditions test-*Ulva* was deployed at three experimental sites in April 2002. Before this it was maintained under low nutrient conditions as described in section 4.3.2. Test-*Ulva* was harvested from the low-nutrient growth chambers and transported to sites in the Whangateau Harbour (WHU), Okura Estuary (OKU) and Motions Creek (MOT) (see next section for site details and description) where it was deployed in surface moorings as described in section 4.3.3. After deployment, sites were subsequently visited at regular intervals for a period of one month and seawater and test-*Ulva* tissue samples collected. Bad weather over the field deployment period meant that samples were missed at the MOT site on two occasions.

### 4.3.7 Field monitoring sites and sampling procedure

A total of 10 field sites were examined in the Auckland Region between 2002 and 2004 (Table 4.1, Figure 4.4 and Figure 4.5. See also Appendix 4.1 for more site details). Based on preliminary seawater analyses three sites were selected as trial sites for this study. These were located in the Whangateau Harbour (WHU), Okura Estuary (OKU) and Motions Creek (MOT) in the Waitemata Harbour. WHU was selected as a low-nitrogen reference site being in a natural harbour with an adjacent, low density human population compared to metropolitan Auckland. The MOT was selected as an urban site in which high (> 40 μM) seawater inorganic nitrogen (mostly nitrate) concentrations had previously been measured. This site as well as being located in Auckland's Waitemata Harbour was also adjacent to Motions Creek which flows though a eutrophic pond at Western Springs and the Auckland Zoo. OKU being on the outskirts of the Auckland metropolitan area was chosen as a site of potential

urban impact but with unknown seawater quality status, although moderate (≈ 10 μM) concentrations had previously been measured in low tide seawater samples.

Most (6) of the ten sites examined in this study were located in the Waitemata Harbour (WH) and represented urban sites (Figure 4.5). According to independent seawater monitoring, the Upper WH harbour had mean seawater TIN concentrations of 4.3 (± 3.9 s.d.) μM compared with 2.0 (± 0.8 s.d.) μM near the low nitrogen reference site (WHU) for the duration of this study (Auckland Regional Council, 2002-2004). Towards the end of the study in the summer of 2004, two additional sites were included. These were located in Alphabet Bay (ALP), near the Leigh Marine Laboratory, and at Puketutu Island (PUK) near the main discharge of the Mangere Wastewater (tertiary) Treatment Plant (MWTP) in the Manukau Harbour (MH) (Figure 4.4). ALP was included as an additional low-N control site while PUK was included as a known sewage wastewater discharge site partly to give more contrasting values of seawater nitrogen loading at the end of the study. All sites were predominantly saline with no large rivers entering them. The lowest salinity values during the survey occurred in the Upper WH sites during periods of heavy rain with values of around 20 ‰.

**Table 4.1.** Locations of 10 sites examined for nutrient loading in the Auckland Region during 2003.

| Site | Location | South | East |
|---|---|---|---|
| 1) LUC = Lucas Creek | Upper Waitemata | 36°46' 21.7" | 174°39' 31.6" |
| 2) UPH = Upper Harbour Bridge | Upper Waitemata | 36°47' 15.5" | 174°40' 03.9" |
| 3) HEN = Henderson Creek | Upper Waitemata | 36°38' 43.9" | 174°39' 38.1" |
| 4) WAU = Whau River | Lower Waitemata | 36°51' 02.6" | 174° 40' 00.3" |
| 5) MEO = Meola Creek | Lower Waitemata | 36°50' 52.7" | 174°42' 26.1" |
| 6) MOT = Motion's Creek | Lower Waitemata | 36°50' 45.6" | 174°42' 58.3" |
| 7) OKU = Okura Estuary | Okura, North Shore | 36°40' 10.1" | 174°43' 31.1" |
| 8) PUK = Puketutu Island | Manukau Harbour | 36°57' 37.2" | 174°44' 02.1" |
| 9) WHU = Whangateau Harbour | Whangateau | 36°19' 03.5" | 174°46' 30.1" |
| 10) ALP = Alphabet Bay, Goat Island | Goat Island, Leigh | 36°16' 04.9" | 174°74' 42.9" |

All WH sites (and PUK in Manukau Harbour) were sampled by boat on the incoming tide and in the same order (MEO, MOT, HEN, UPH and LUC) to standardise sampling time with respect to low tide. Sampling all WH sites was done by boat because most of these sites were situated near deep mud channels. Prior to the commencement of the main survey in December 2002 the WHU, OKU and MOT sites had been sampled on a monthly basis at low tide on foot. From December 2002 to end of the survey in February 2004, sampling at WHU and OKU was continued on foot while the MOT site was sampled usually just after low tide. Since it took about 30 minutes on average to process samples and travel to the next site it usually took about 3 to 4 hours to complete all WH sites. All moorings were located in, or as near to as possible (without causing a navigational hazard) the main channels at each site. In addition, sites were chosen such that chambers were always in water (even at spring low tides) so that test-algae never became desiccated.

Prior to each monthly visit test-*Ulva* was treated as described in Section 4.3.2 and then deployed at field sites for one month. At each monthly visit algal samples were removed from enclosures, the frames cleaned of any fouling organisms, and the netting replaced. Unless otherwise specified test-*Ulva* was left in the field enclosures for one month and then sampled as follows. *Ulva* tissue was collected from each of three replicate moorings at each site. Only large thalli that were not attached to the enclosure frame were sampled from the upper, well-lit half of the enclosures. This was done to minimise the chance of sampling thalli that had been shaded by others. Tissue sampling was then conducted as described in sections 4.3.10 to 4.3.14 below. Approximately 1 g of new test-*Ulva* was then redeployed in the enclosures for the next month. This was repeated every month until the beginning of 2004.

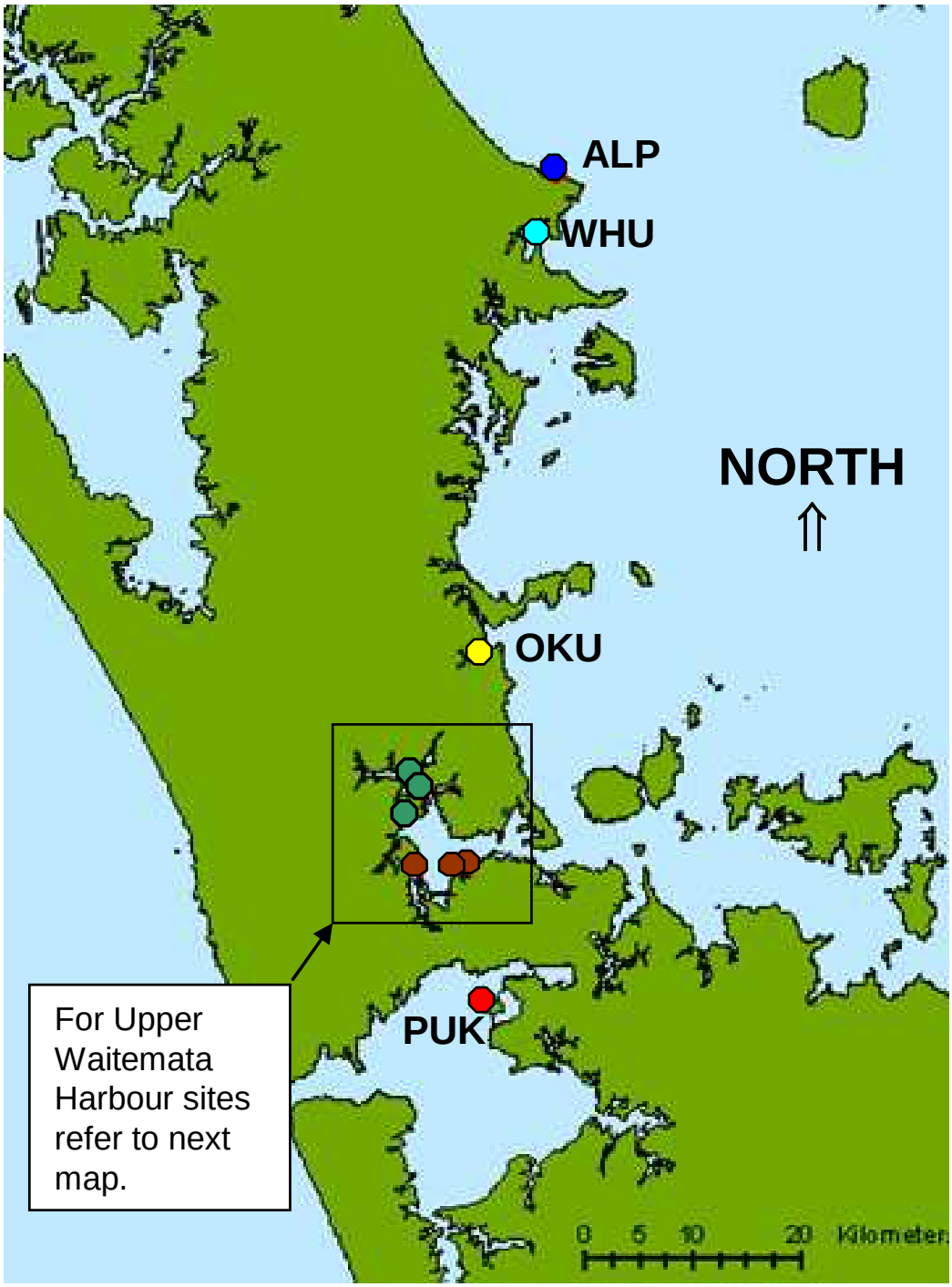



**Figure 4.4.** Location of surface moorings at ten sites in the Auckland Region, New Zealand.

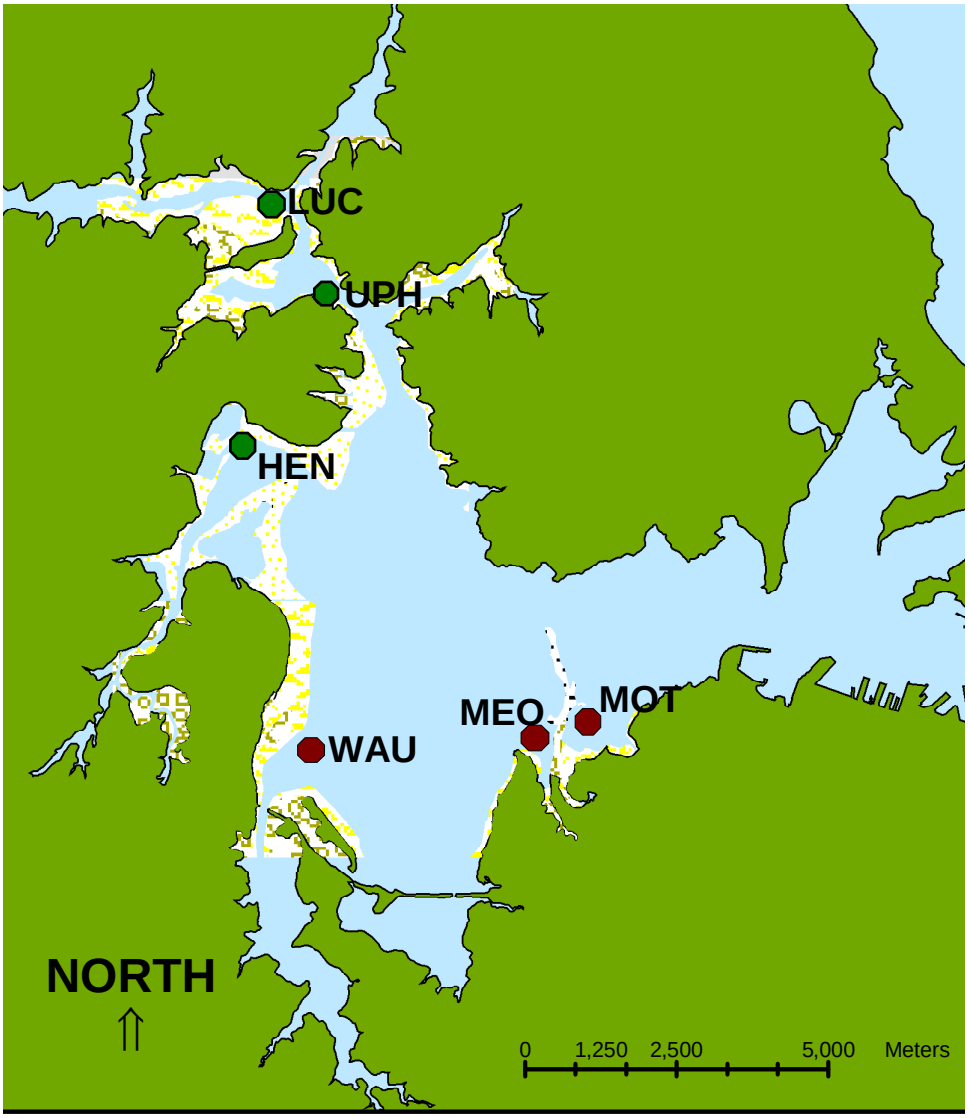



**Figure 4.5.** Location of surface moorings in three lower and three upper Waitemata Harbour sites.

### 4.4.8 Experiment 2. Experimental validation of responses of test-*Ulva* to constant seawater nitrogen concentration.

In order to evaluate the responses of test-*Ulva* nitrogen indices to seawater nitrogen concentration, algae were maintained in seawater with different constant concentrations of nitrogen for 10 days. Three separate experiments were conducted over the last half of 2003 to capture at least some of the seasonal influences on N-indices in test-*Ulva*. Eight ammonium concentrations (including a control with only natural, low-nitrogen seawater) were chosen: 2, 4, 6, 8, 10, 15 and 20 μM (plus

ambient nutrient concentrations). Note that previous experiments (see Chapter Three, Figure 3.15) comparing the biochemical responses of test-*Ulva* to nitrate versus ammonium additions showed that there were no strong differences in gross N-indices (total TN, TChl and FAA content) in response to these nitrogen sources. Phosphorus was added to give a constant N : P ratio of 10 : 1. A total of three separate experiments were conducted during winter, spring and summer in 2003. During the three experiments, the ambient Goat Island seawater TIN concentration ranged from 1.3 ± 0.1 μM to 1.9 ± 0.7 μM, with a mean concentration of 1.6 ± 0.2 μM.

### 4.4.9 Experiment 3. Experimental validation of responses of test-*Ulva* to pulsed versus constant nitrogen concentration.

At the end of the survey a third experiment was conducted to evaluate the responses of test-*Ulva* to pulsed versus constant nitrogen supply at two average nitrogen (ammonium) concentrations. Nitrogen (and phosphorus to give a N : P = 10 : 1, as described in Experiment 2) were supplied to test-*Ulva* at two concentrations (5 μM and 10 μM), either as a constant supply or as a 12 hourly (to simulate tidal flux) pulsed supply (i.e. with low concentrations for 6 hours followed by high concentrations for 6 hours) (see Figure 4.6 for nutrient addition regimes). This was achieved by using two peristaltic pumps, one running continuously supplying constant concentrations, the other cycled at 6-hourly intervals supplying pulsed additions which were ‘added’ to constant concentrations. The total N loading was the same for both treatments. Seawater nutrient concentrations were analysed at regular intervals and *Ulva* was sampled after three weeks enrichment. As with Experiment 2 three biochemical responses in test-*Ulva* were examined; TN, TChl and FAA content. Sample collection was timed to coincide with peak concentration to simulate sampling at low tide.

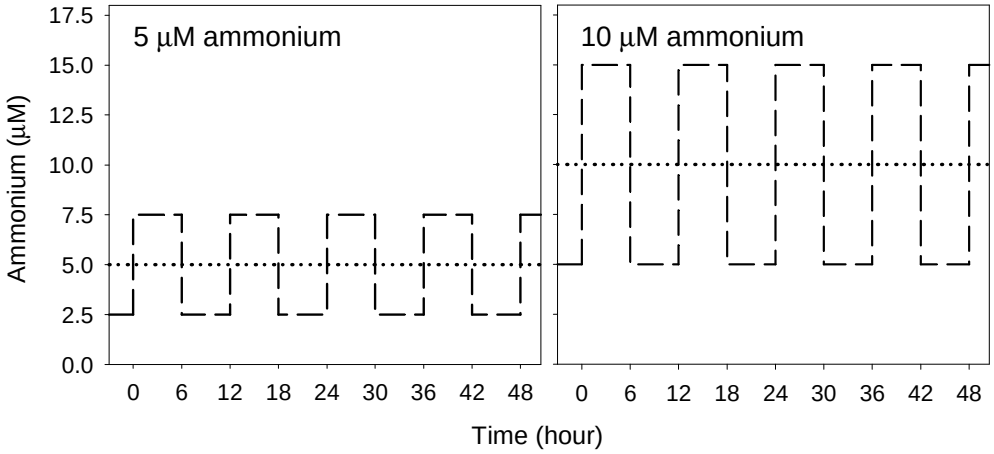



**Figure 4.6.** Conceptual representation of test-*Ulva* enrichment experiment using two ammonium concentrations (5 μM and 10 μM) supplied to test-*Ulva* either as constant, or as pulsed (6 hrs at 0.5 × constant value followed by 6 hrs at 1.5 × constant value) ammonium additions. Dotted lines represent constant ammonium additions (constant concentration) while the dashed lines represent the pulsed ammonium additions (pulsed concentration).

***Analytical***

### 4.3.10 Free amino acids

From either growth enclosures, or experimental chambers, *Ulva* thalli were collected and 3 - 4 g of tissue randomly sub-sampled as described in Chapter Two using the method of Barr and Rees (2000). Amino acid samples were extracted and analysed from 1 g fresh weight sub-samples of *Ulva* tissue as described in Chapter Two, and for field samples with the following modifications. Amino acid extraction in the field was conducted using pre-prepared field kits comprised of vials with pre-measured volumes (5 mL) of either 1M perchloric acid or 1M KOH/0.2 M MOPS. Extracted amino acid samples were kept on ice during transport back to the Leigh Marine Laboratory where samples were stored at - 80 ˚C for later HPLC analysis of amino acid content. The extracted tissue was kept for later determination of dry weight by first rinsing with distilled water and then drying to a constant weight at 65 ˚C. Values for all amino acids (and their ratios) are expressed as μmol N · g $DW^{-1}$.

The remaining (2 - 3 g) tissue samples were also stored on ice in labelled zip-lock bags for return to the Leigh Marine Laboratory where they were frozen at -18 ˚C and later analysed for chlorophyll and tissue nitrogen content.

### 4.3.11 Chlorophyll

After thawing, chlorophyll was extracted and measured as described in Chapter Two. Values are expressed as mg · g $DW^{-1}$.

### 4.3.12 Tissue nitrogen content

After thawing, tissue nitrogen and $\delta^{15}N$ content was measured as described in Chapter Two.

### 4.3.13 Tissue metal content

Metal analysis was conducted on dried and ground whole tissue samples by AgResearch Limited, Hamilton. Samples for heavy metal analysis were prepared separately from those described for the other metabolic samples above to avoid metal contamination. Tissue samples were randomly sub-sampled from individual thalli by hand using latex gloves. Analysis was conducted on a Varian Vista AX CCD Simultaneous Inductively Coupled Plasma-Atomic Emission Spectrophotometer (ICP-AES). Samples were quantified against 1000 ppm atomic absorption standard solutions.

### 4.3.14 Growth

Growth rate in field deployed test-*Ulva* was measured as the change in fresh weight biomass over the period of deployment. Prior to weighing, excess surface water was removed by vigorous shaking in a mesh bag. Growth was expressed as daily specific growth rates, calculated as:

$$\text{Growth} = \ln \frac{\text{final mass / initial mass}}{\text{time interval (day)}}$$

### 4.3.15 Seawater sampling and nutrient analyses

Seawater samples were taken at the same time and location as algal tissue samples. Samples were kept chilled on ice and then stored frozen at -18 $^{o}C$ until they were analysed. These were later thawed and analysed for ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$) and phosphate ($PO_4^{3-}$) with reference to standard curves containing known concentrations of nutrients. Ammonium was determined as described by

Koroleff (1983a) and nitrite by Parsons *et al* (1984). Nitrate was reduced to nitrite by passing 20 ml of seawater sample through a cadmium column, and then determined using the method described by Parsons *et al* (1984). Phosphate was determined as described by Koroleff (1983b). Total inorganic nitrogen (TIN) concentrations were calculated as the sum of $NH_4^+$, $NO_2^-$ and $NO_3^-$. The nitrogen : phosphorus (N : P) ratio was calculated as TIN / $PO_4^{3-}$.

### 4.3.16 Statistical analysis

Regression lines were fitted by ordinary least squares in SigmaStat 3.1. Means were compared using a two-way or three-way general linear model (analyses of variance). The Holm-Sidak method was used to compare among means. Where assumptions of normality or equal variance were not met by the data then comparisons were made using Kruskal-Wallis analysis of variance on ranks.

## 4.4 Results

### 4.4.1 Experiment 1. Field equilibration of test-*Ulva*.

Chlorophyll was used as a proxy for nitrogen content for the duration of the following experiment (see Chapter Three, Figure 3.21) and extracted from small sub-sampled portions of test-*Ulva* at regular intervals during the whole experiment. After 30 days of culturing in low nutrient conditions at the laboratory, test-*Ulva* was noticeably paler compared to starting conditions and contained lower total chlorophyll (*a* + *b*) (TChl) levels (0.8 ± 0.0 mg · g $DW^{-1}$) than it did at collection (5.4 ± 0.2 mg · g $DW^{-1}$) (Figure 4.7). Conversely, after 30 days of field deployment TChl at the three trial sites increased to relatively stable plateaus after about three weeks (Figure 4.7, A). The plateaux in total chlorophyll concentrations attained by test-*Ulva* were different for each site, with values for MOT being greatest and least at WHU. In contrast, seawater TIN concentrations were variable, but slightly higher at OKU compared with MOT. WHU had the lowest average low-tide TIN concentrations compared with the other two sites (Figure 4.7, D). When nutrient data were examined in a longer

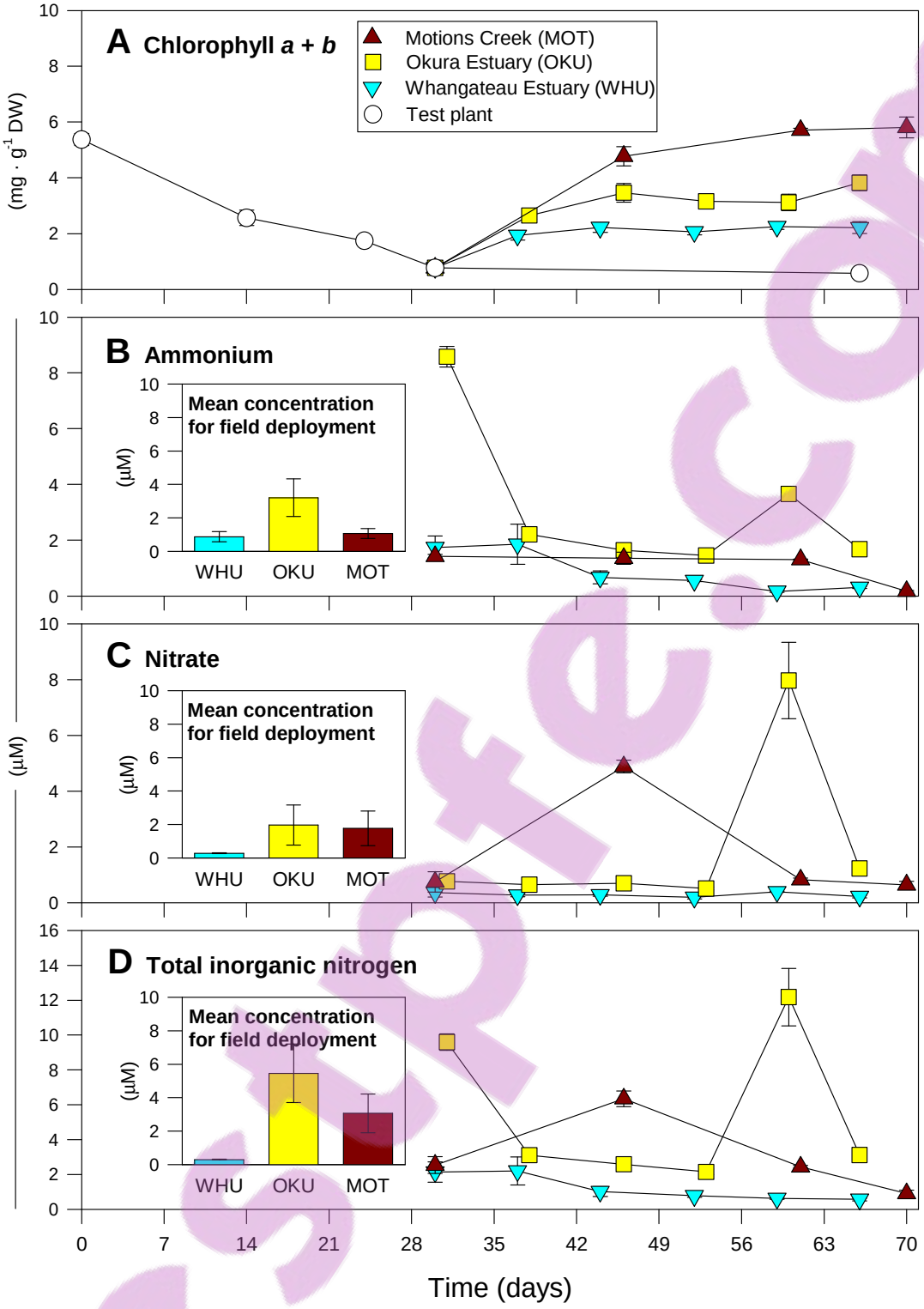



**Figure 4.7.** Changes in chlorophyll *a* + *b* content in test-*Ulva* plants under low nutrient conditions and then during deployment at the Whangateau Harbour (WHU), Okura Estuary (OKU) and Motions Creek (MOT). Values are means ± standard errors for three separate replicates. Inset plots are average (± standard errors) for all seawater samples taken at each site during the deployment period.

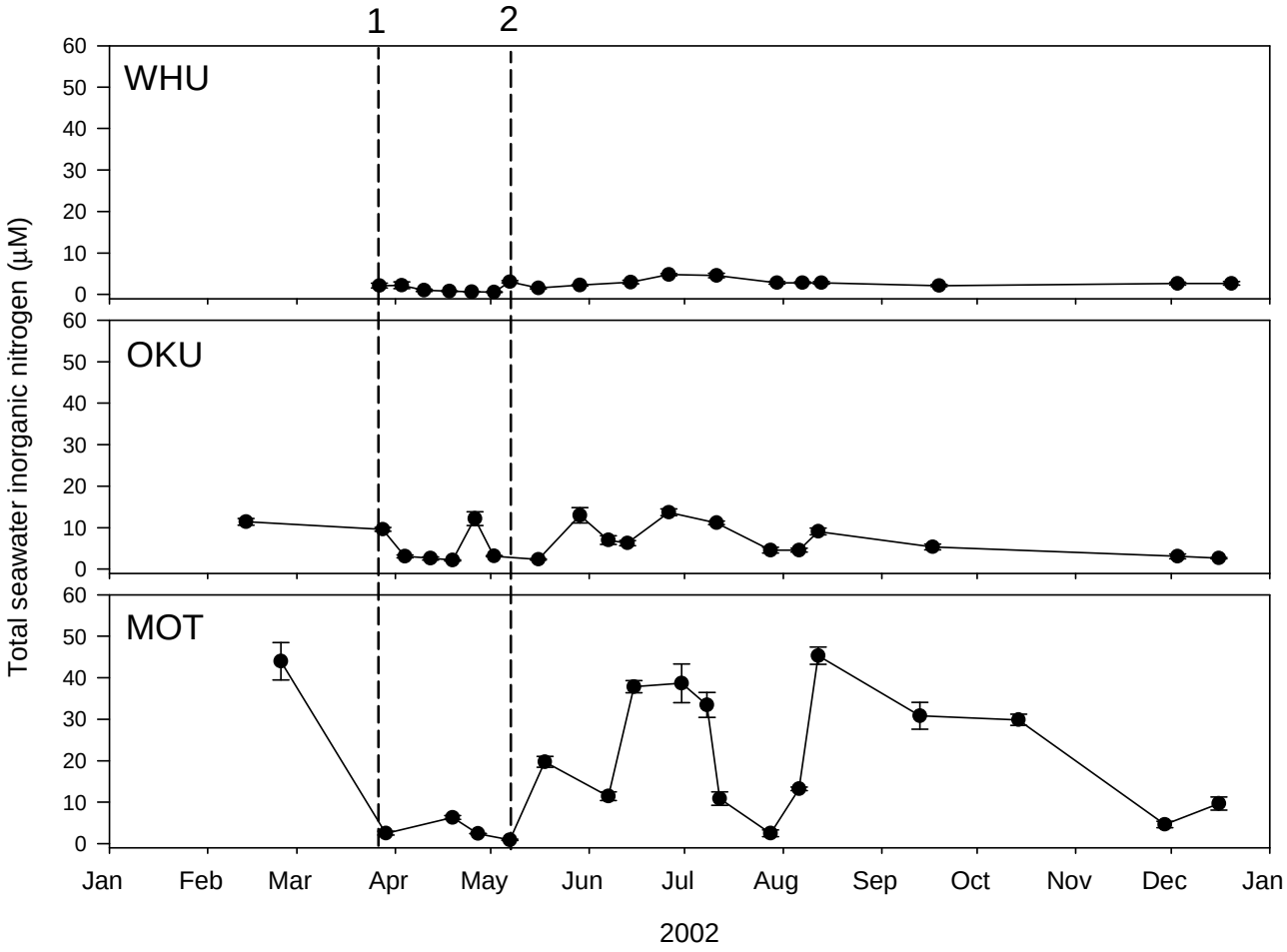



**Figure 4.8.** Comparison of changes in seawater total inorganic nitrogen concentrations with time at three sites: Whangateau Harbour (WHU), Okura Estuary (OKU) and Motions Creek (MOT) over one year (2002 to 2003). Values are means ± standard errors for three separate replicates. The dashed lines indicate when Experiment 1 was conducted.

term (one year) context it was apparent that MOT site was highly variable often with very high (up to 44.0 µM) TIN concentrations (comprised mainly of $NO_3^+$) compared with either OKU or WHU (Figure 4.8). Of the three sites WHU had consistently lower seawater TIN concentrations.

By the end of deployment WHU had test-*Ulva* with the lowest levels of all indices while MOT had the highest (Figure 4.9). In addition, the unknown amino acid $U2^{9.48}$ showed the greatest range of values in test-*Ulva* between the three sites and was clearly the highest at MOT. Despite OKU having slightly higher average nitrogen

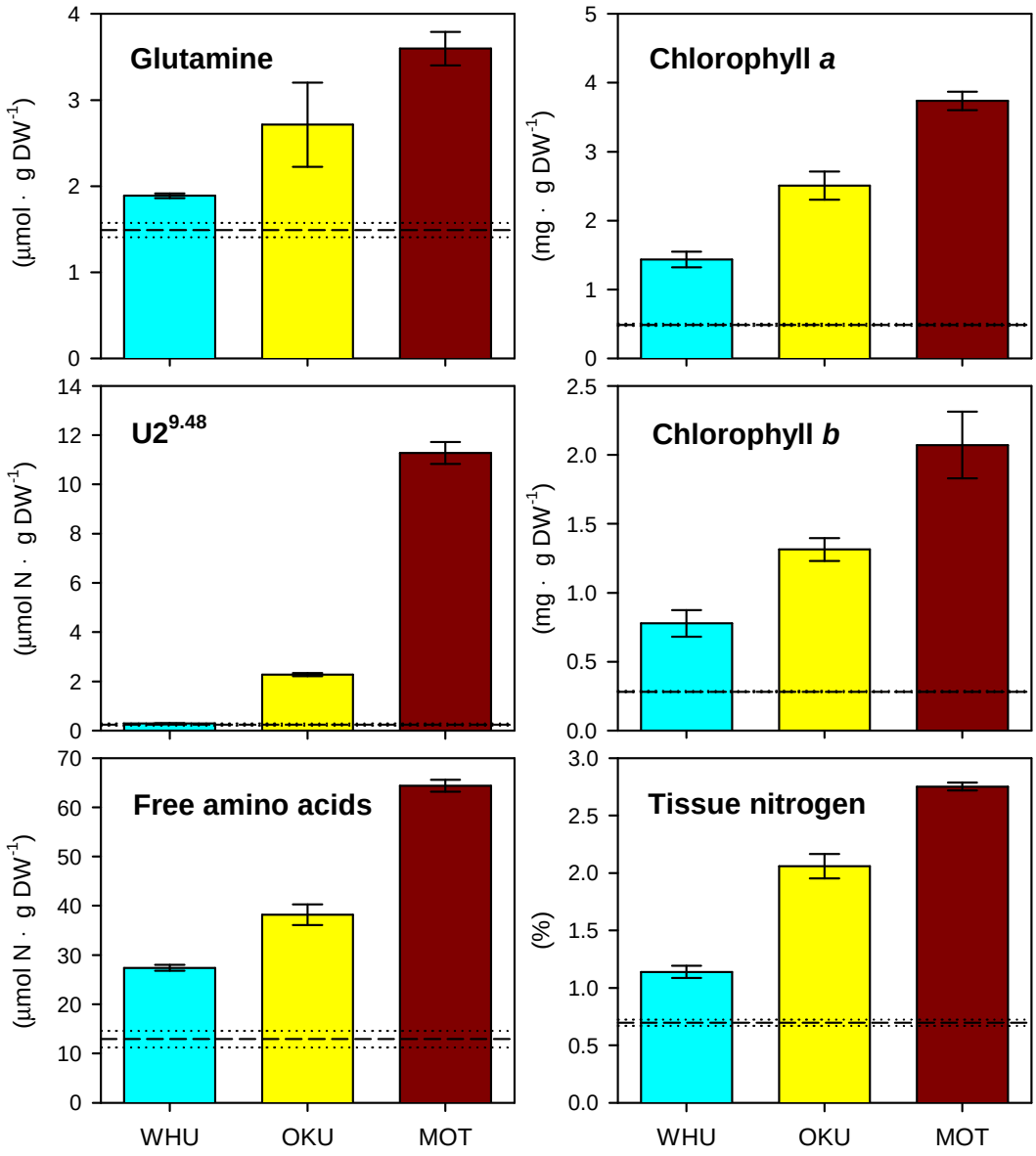



**Figure 4.9.** Comparison of selected N-indices in test-*Ulva* after 30 days field deployment at sites in the Whangateau Harbour (WHU), Okura Estuary (OKU) and Motions Creek (MOT). Values are means ± standard errors for three separate replicates. Dashed and dotted lines indicate means ± standard errors, respectively, for levels of N-status indices at the beginning of field deployment.

concentrations compared with MOT seawater, test-*Ulva* from MOT had the highest levels of N-indices while OKU had intermediate levels.

***Field-based monitoring (2003-2004)***

### 4.4.2 Monitoring seawater inorganic nitrogen concentration using test-*Ulva*.

Seven sites were examined from December 2002 to February 2004. These included the three trial sites, WHU, OKU and MOT, plus four additional Waitemata Harbour (WH) sites: MEO, HEN, UPH and LUC. Three additional sites were also added in the summer of 2004; ALP (exposed open coastal) WAU (lower WH) and PUK (wastewater) (see section 4.3.7). These three sites are not presented in time series data below because they were only examined at the end in summer 2004.

All sites also showed increases in both seawater TIN concentrations and N-indices in test-*Ulva* in the winter (Figure 4.10). Moreover, of the N-indices examined, the total free amino acid (FAA) pool showed reasonable correlations with seawater TIN concentrations during 2003, particularly at MEO (Figure 4.11). However, there were clearly different regression relationships between the sites with lower regression slopes in the plots of WHU, OKU, MOT and MEO compared with the Upper WH sites of HEN, UPH and LUC (Figure 4.11). The WHU, OKU, MOT and MEO sites were all sampled either at, or close, to low tide compared with the Upper WH sites sampled later in the flood tide. The higher seawater TIN concentrations relative to FAA content in test-*Ulva* from OKU, MOT and MEO possibly reflected a greater contribution from recent low tide nutrient fluxes (from either fresh water sources (e.g., from the adjacent creeks in the case of MEO and MOT) or possibly from ammonium efflux from exposed sediments at low tide). On one occasion at OKU, seawater samples were taken at high tide resulting in 4.7-fold lower seawater TIN concentrations (as indicated by arrows in the top two middle plots of Figure 4.10) compared with samples taken six hours previously at low tide. This presumably resulted from tidal dilution with 'new' water during the flood tide. In contrast to this, seawater samples taken at the UPH site close to the end of the study showed little change in nutrient concentration over a tidal cycle (data not shown). This possibly reflects a more homogeneous water column in this part of the Upper Waitemata harbour. It should be noted that the Upper Waitemata has a large tidal excursion of about 3 km and a relatively long retention time (9 – 11 tidal cycles. Auckland Regional Authority, 1983) compared with sites in Lower WH, and at WHU and OKU.

Out of the seven long-term sites, WHU site had both consistently low TIN concentrations (0.9 to 4.0 μM) and N-indices in test-*Ulva* (e.g. FAA levels ranging from 27.4 to 68.9 μmol N · g $DW^{-1}$) throughout the year (Figure 4.10). OKU had test-*Ulva* with N-indices that were intermediate between WHU and MOT, but often with high (up to 11.5 μM) seawater TIN concentrations (Figure 4.10 and Figure 4.11) and consistently high ammonium concentrations at low tide during winter. Compared with WHU, MOT and the other WH sites had higher, but variable (at monthly scales) seawater TIN concentrations. However, test-*Ulva* from these sites clearly had higher levels of both individual FAA and total FAA (Figure 4.10). The most contrasting differences in N-indices between these sites were in levels of glutamine, $U2^{9.48}$, proline and to a lesser extent the total FAA pool (Figure 4.10). In addition, seawater nitrogen concentrations and N-indices in test-*Ulva* from WH sites were generally similar to each other throughout the year. A prominent feature in data from the WH sites was that both seawater TIN and amino acids in test-*Ulva*, particularly glutamine, showed pronounced peaks in both July and late October (Figure 4.10). In addition, the Upper WH sites (HEN, UPH and LUC) showed very similar trends in seawater TIN and TN content in test-*Ulva*. It is possible that peaks in seawater TIN concentrations at these sites related to higher rainfall at these times since seawater TIN concentrations showed broad negative correlations with salinity (Auckland Regional Council, 2002-2004) (data not shown). Moreover, runoff data from HEN (Henderson River, NIWA climate database 2003) suggested a link between N-indices in test-*Ulva* and nitrogen which was possibly derived from runoff supplied to the Harbour, at least at this site (see Appendix 4.2). It was also noted that there was a pronounced decrease in TN content in test-*Ulva* at HEN, UPH and LUC, with values of close to 2 % in late November at these three sites (Figure 4.10). This may have related to increases in seawater chlorophyll that occurred around this time (Auckland Regional Council, 2002-2004), reflecting that higher densities of phytoplankton in the water column may have out-competed test-*Ulva* for available nitrogen at these sites.

Although there were seasonal (winter) increases in N-indices (e.g., TChl and TN) in test-*Ulva* from all (7) sites examined through 2003, contrasting differences in N-indices between sites were apparent around the summer months (e.g., between WHU and Upper WH sites) (Figure 4.10). However, the greatest contrast in levels of any N-index between all sites in this study (note that three additional sites [ALP, WAU and

PUK] were added were added as outlined previously) was seen in FAA content in the summer (Figure 4.12, A). In addition, it was apparent that there were significant differences in FAA content in test-*Ulva* from many sites relative to the WHU control site. However, over the same period of time the only significant difference in seawater TIN concentration with respect to the WHU control site was at the PUK wastewater site (Figure 4.12, B).

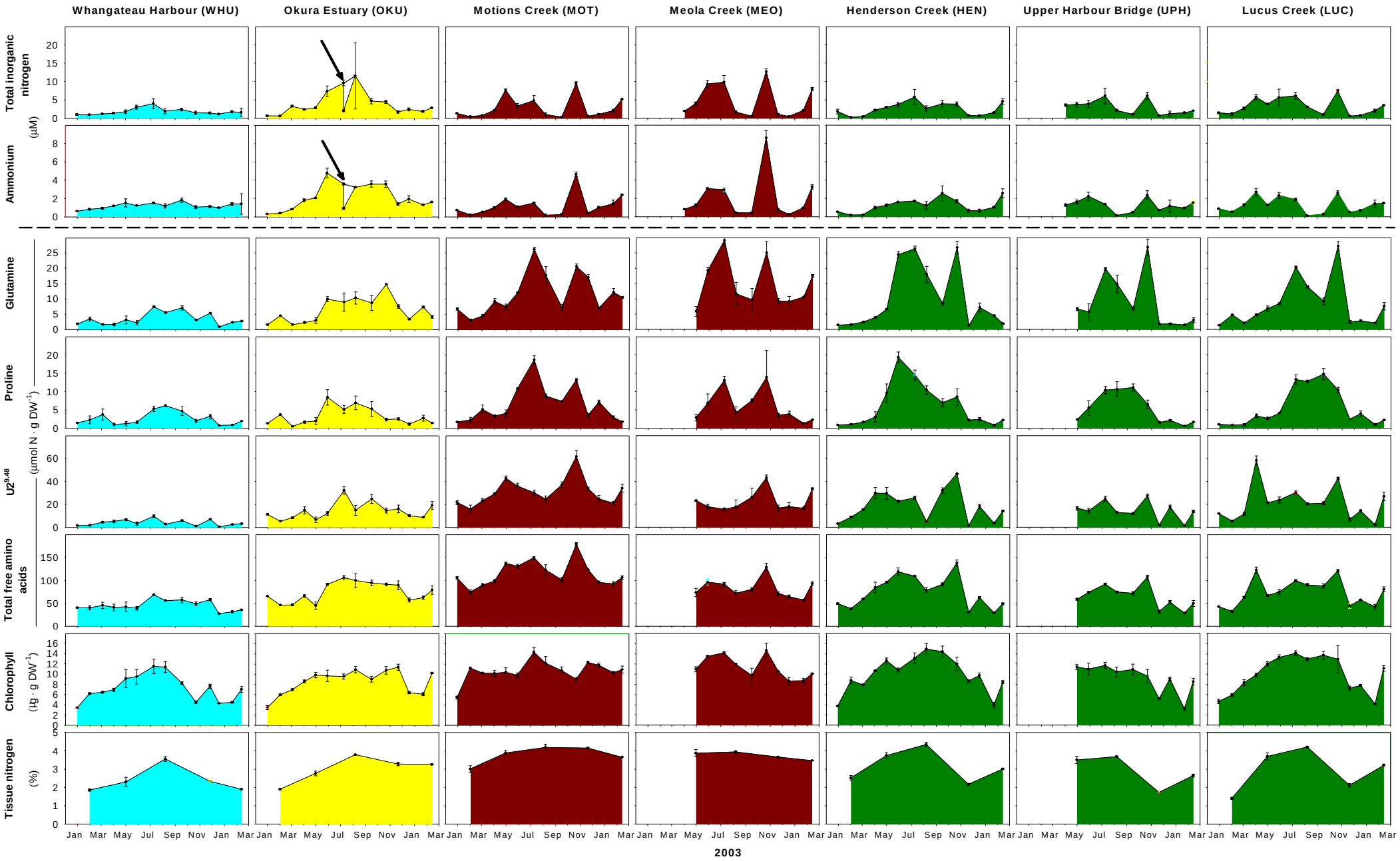

Whangateau Harbour (WHU)
Okura Estuary (OKU)
Motions Creek (MOT)
Meola Creek (MEO)
Henderson Creek (HEN)
Upper Harbour Bridge (UPH)
Lucus Creek (LUC)
Total inorganic nitrogen (µM)
Ammonium
Glutamine
Proline
U2[9,48] (µmol N · g DW-1)
Total free amino acids
Chlorophyll (µg · g DW-1)
Tissue nitrogen (%)
Jan Mar May Jul Sep Nov Jan Mar
2003

**Figure 4.10.** Trends in seawater total inorganic nitrogen and ammonium concentrations (top two rows of plots) and N-indices (bottom six rows of plots) in test-*Ulva* deployed at surface moorings at three sites in Auckland region in 2003 / 2004. Values are means ± standard errors for 3 replicate stations at each site. Green fills indicate Upper Waitemata Harbour sites. Note that arrows in the plots for OKU seawater nutrient data in the top two rows indicate when both low tide and high tide seawater samples were taken in July 2003.

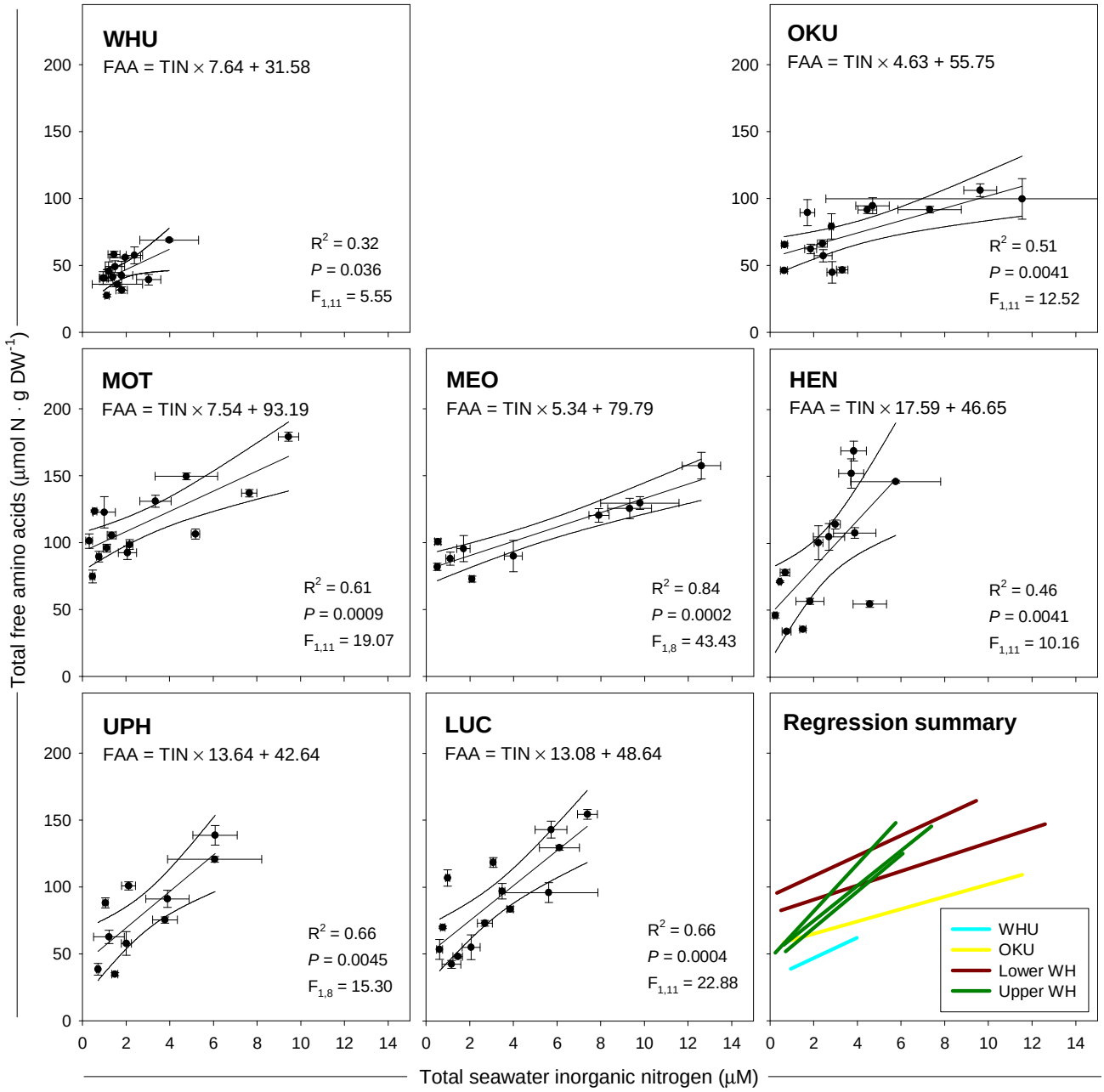



**Figure 4.11.** Changes in free amino content with changes in seawater total inorganic nitrogen concentrations in test-*Ulva* deployed at surface moorings at seven sites around Auckland in summer 2003 to summer 2004. Note that in the case of the Waitemata Harbour sites they are presented going up into the harbour in order: MOT, MEO, HEN, UPH and LUC. Values are means ± standard errors for 3 replicate stations. A summary of regression lines are presented in the bottom right-hand plot.

### 4.4.3 Experiment 2. Experimental validation of responses of test-*Ulva* to constant seawater nitrogen concentration.

Test-*Ulva* was treated the same way as that deployed in the field (i.e., by first depleting its nitrogen stores as described in section 4.3.2), but was then enriched with a range of constant ammonium concentrations for at least nine days. After enrichment, TN, TChl and FAA content in test-*Ulva* showed a saturating response to seawater nitrogen concentration (Figure 4.13). Out of these three indices, the FAA pool showed the greatest range (6.3-fold) of values (29.2 ± 3.7 μmol · g $DW^{-1}$ and 185.7 ± 7.7 μmol · g $DW^{-1}$) in response to increasing nitrogen concentration compared with either TChl (2.7-fold) or TN (2.3-fold) (Figure 4.13). Individual amino acids including: glutamine, glutamate, asparagine, aspartate, proline and $U2^{9.48}$ were also examined (note: as described in Chapter Three, section 3.3.6, histidine was not included in this analysis because it eluted closely to another unknown peak that was a consistent feature in test-*Ulva*). Most of these amino acids showed similar saturating responses to increases in ammonium concentration as did the total FAA pool (Appendix 4.3). However, some individual amino acids, such as glutamate and asparagine, were accumulated at lower levels (< 10 μmol · g $DW^{-1}$) while others such as glutamine, $U2^{9.48}$ and proline were accumulated at higher levels (72.8 ± 4.6 μmol · g $DW^{-1}$, 49.2 ± 5.1 μmol · g $DW^{-1}$ and 14.9 ± 5.1 μmol · g $DW^{-1}$, respectively) in test-*Ulva* tissues supplied with 20 μM ammonium.

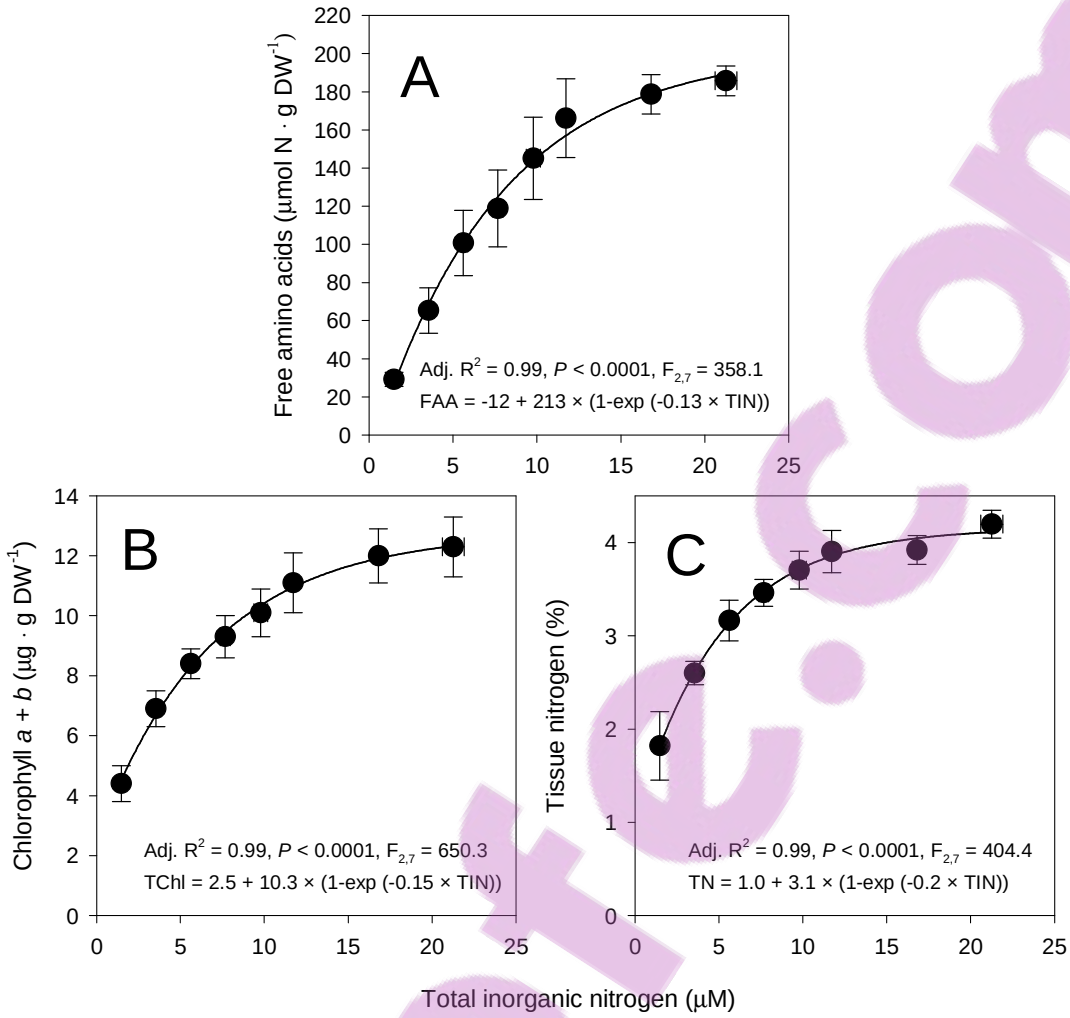



**Figure 4.12.** Change in levels of (**A**) total free amino acids, (**B**) total chlorophyll and (**C**) total tissue nitrogen in test-*Ulva* with change in ammonium concentration. Values are means ± standard errors for 3 separate experiments. Regression curves are fitted using SigmaStat 9 with an exponential rise to maximum model.

### 4.4.4 Experiment 3. Experimental validation of responses of test-*Ulva* to pulsed versus constant nitrogen concentration.

A similar experiment to the one above was conducted to evaluate whether N-indices in test-*Ulva* could integrate average nitrogen concentrations supplied either constantly or as tidal scale pulses. From seawater nutrient results it was concluded that there was obvious potential for instantaneous seawater samples collected at low tide to over-represent tidal average concentrations as was probably the case at OKU (Figure 4.10). It was equally possible that seawater samples collected either before or

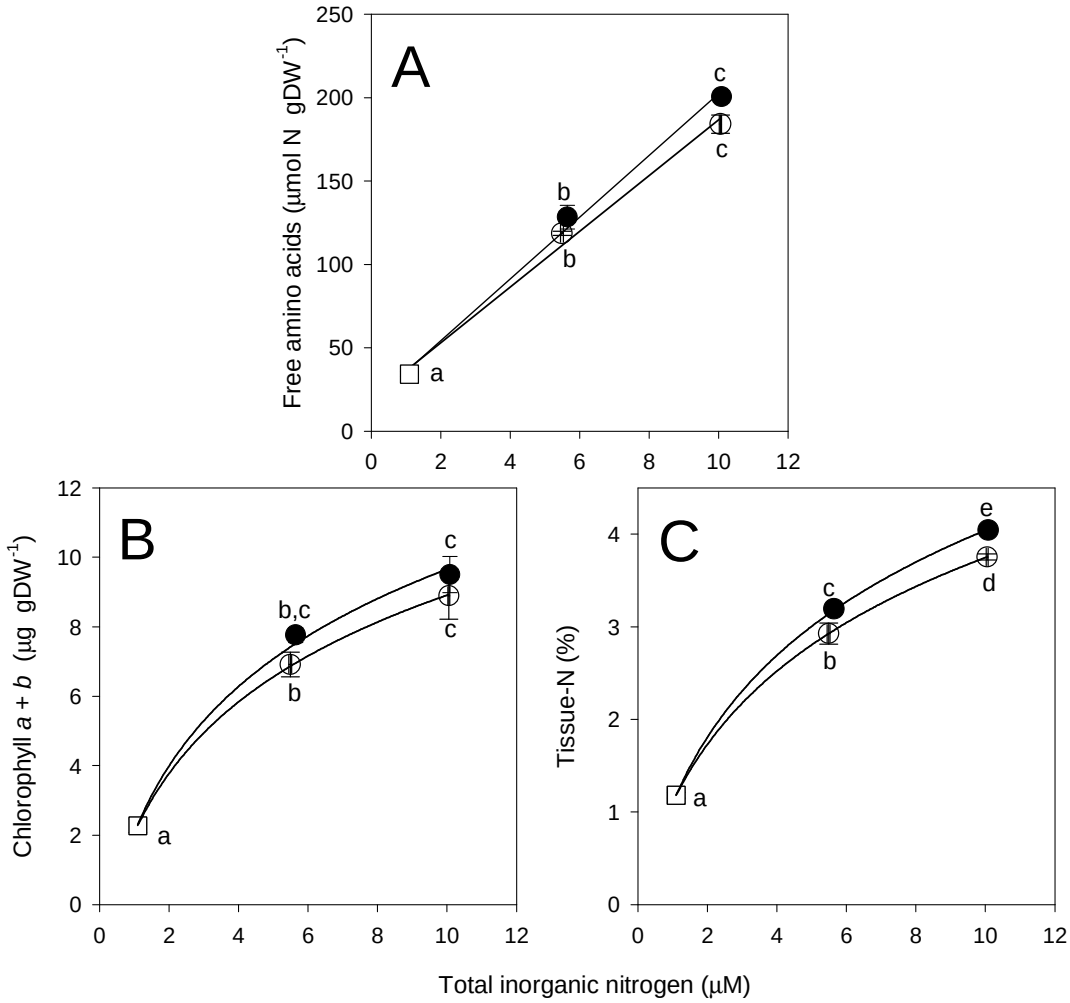



**Figure 4.13.** Change in total (**A**) free amino acids, (**B**) chlorophyll and (**C**) tissue nitrogen in test-*Ulva* with change in average ammonium concentration. Ammonium was supplied as either a constant concentration (filled circles) or as a continuous pulse train of two alternating concentrations (6 hrs at 0.5 × constant value followed by 6 hrs at 1.5 × constant value) (open circles). Control treatments were given no added nutrients (open square). Values are means ± standard errors for 3 replicates. One-way ANOVA: A, $F = 214.6$, $P < 0.001$, $n = 3$; B, $F = 45.7$, $P < 0.001$, $n = 3$; C, $F = 266.5$, $P < 0.001$, $n = 3$. Data points labelled with the same lower case letter indicate that test-*Ulva* nitrogen indices do not differ significantly ($P > 0.05$) according to the Holm-Sidak procedure for multiple pair-wise comparisons. Note that a linear regression model was fitted to both curves in Figure A, while the same exponential rise to maximum regression model that were used in Figure 4.12 was applied to the curves Figures B and C.

after low tide would 'miss' low-tide pulses while high tide sampling could under-represent tidal average concentrations because of tidal dilution. It was known from

results of Chapter Three (Figure 3.4) and from Figure 4.7 earlier, that chlorophyll (and therefore probably tissue nitrogen) changed over a period of 2 to 3 weeks while the total FAA pool changed over much shorter time scales (up to a few days) (Figure 3.5, A) in response to nitrogen addition. In this experiment test-*Ulva* under enrichment were sampled during the high nitrogen pulse phase to simulate collection in the field at low tide. None of the three indices examined strongly differentiated between pulsed and constant nitrogen supply although, as with the previous experiment, it was noticeable that TChl and TN, compared with FAA, showed saturating responses over this range of concentrations. Therefore, it was concluded that values of the total pool of FAA, TChl and TN content could integrate the average nitrogen concentration (for both 5 μM and 10 μM ammonium) regardless of whether it was supplied constantly or as tidal scale pulses of nitrogen (Figure 4.14).

### 4.4.5 Season and nitrogen

Seasonal variation in nitrogen content in test-*Ulva* was examined using all data by correlation with temperature and light data. Irradiance was taken as total light energy ($MJ \cdot day^{-1}$) averaged for the 30 days prior to sample collection. Irradiance data for the individual sites was obtained from the nearest (< 10 km distance) NIWA (National Institute for Water and Atmosphere) weather station. Seawater temperature was taken with a thermometer at the time of sample collection. From regression analysis the lowest average daily irradiance for all sites occurred in July while the lowest seawater temperatures occurred in August (Figure 4.15).

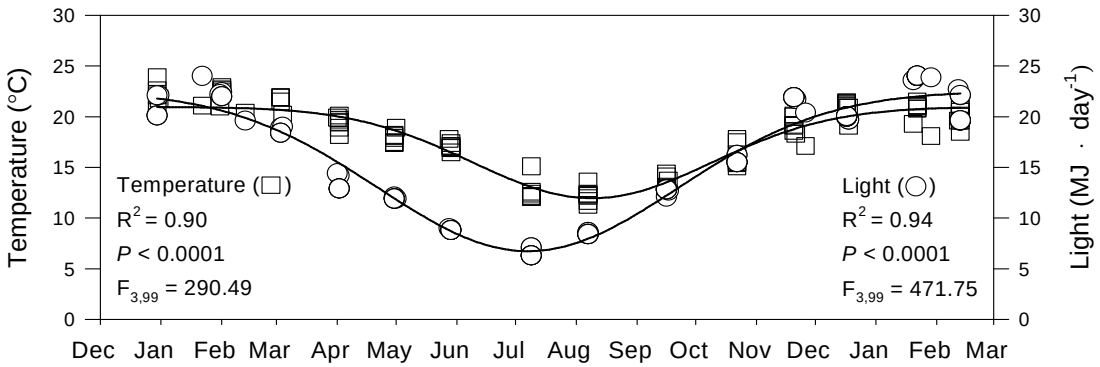



**Figure 4.14.** Changes in temperature (squares) and monthly mean irradiance (circles) for monitoring sites during 2003. Regression analysis made using the peak curve fitting function in SigmaStat 9.

Multiple linear regression analysis was conducted using all data from the survey to examine the effects of seawater inorganic nitrogen, seawater temperature and surface light on three N-indices (FAA, TChl and TN) in test-*Ulva*. Although correlations were weak in all three models, total seawater inorganic nitrogen concentration showed a significant correlation with FAA, TChl and TN (Table 4.2). According to these results the only other abiotic factor that correlated with N-indices in test-*Ulva*, other than seawater TIN concentration, was surface irradiance which showed a significant correlation with TChl (Table 4.2, B).

**Table 4.2.** Multiple-linear regression models for abiotic factors versus test-*Ulva* total (**A**) tissue nitrogen, (**B**) chlorophyll and (**C**) free amino acid content for all data from December 2002 to February 2004. Abiotic factors: TIN = total seawater inorganic nitrogen, Temp = seawater temperature (˚C) and Light = surface radiation (MJ · $d^{-1}$) measured in air. Tissue-N indices; TN = tissue-N (%), TChl = chlorophyll *a* + *b* (µg · g $DW^{-1}$) and FAA = free amino acids (µmol N · g $DW^{-1}$).

**A. Total tissue nitrogen**

$$TN = 4.9 + (0.055 \times TIN) - (0.064 \times Temp) - (0.052 \times Light)$$

$R^2 = 0.46$. Analysis of Variance: $F_{3,37} = 9.55$, $P < 0.001$
*P*-values for independent variables: TIN < 0.001, Temp = 0.358, Light = 0.204

**B. Total chlorophyll**

$$TChl = 15.4 + (0.15 \times TIN) - (0.13 \times Temp) - (0.27 \times Light)$$

$R^2 = 0.47$. Analysis of Variance: $F_{3,97} = 28.22$, $P < 0.001$
*P*-values for independent variables: TIN = 0.002, Temp = 0.26, Light < 0.001

**C. Total free amino acids**

$$FAA = 127.1 + (4.4 \times TIN) - (1.8 \times Temp) - (1.5 \times Light)$$

$R^2 = 0.50$. Analysis of Variance: $F_{3,97} = 31.86$, $P < 0.001$
*P*-values for independent variables: TIN < 0.001, Temp = 0.225, Light = 0.089

Single-linear regression relationships were also examined individually between test-*Ulva* N-indices, and seawater inorganic nitrogen, temperature and surface light data. Although correlations were again weak between individual factors and response variables in test-*Ulva*, both TN and TChl were both individually better correlated with light and temperature than they were with seawater TIN concentration (Table 4.3). More of the variation in both TN and TChl ($R^2$ = 0.26 and $R^2$ = 0.41, respectively) was explained by light than either temperature or seawater TIN concentration. Conversely FAA was better correlated with seawater TIN concentration than either light or temperature (Table 4.3). Finally, it is worth noting that the slope of all relationships between test-*Ulva* N-indices and external abiotic variables was significant (Table 4.3).

**Table 4.3.** Single linear regression statistics of abiotic factors versus test-*Ulva* total (**A**) tissue nitrogen, (**B**) chlorophyll and (**C**) free amino acid content for all data from December 2002 to February 2004. Abiotic factors: TIN = total seawater inorganic nitrogen, Temp = seawater temperature (˚C) and Light = surface radiation (MJ · $d^{-1}$) measured in air. Tissue-N indices; TN = tissue-N (%), TChl = chlorophyll *a* + *b* (µg · g $DW^{-1}$) and FAA = free amino acids (µmol N · g $DW^{-1}$).

| **A. Total tissue nitrogen** | **$R^2$** | ***P*** | **DF** | **F** |
|---|---|---|---|---|
| TN = 4.6 - (0.08 × Light) | 0.26 | 0.001 | 1,37 | 12.44 |
| TN = 5.5 - (0.13 × Temp) | 0.21 | 0.003 | 1,37 | 9.77 |
| TN = 2.9 + (0.05 × TIN) | 0.18 | 0.007 | 1,37 | 8.12 |
| **B. Total Chlorophyll** | **$R^2$** | ***P*** | **DF** | **F** |
| TChl = 15.0 - (0.35 × Light) | 0.41 | <0.001 | 1,97 | 66.53 |
| TChl = 18.7 - (0.52 × Temp) | 0.31 | <0.001 | 1,97 | 43.98 |
| TChl = 8.5 + (0.21 × TIN) | 0.12 | <0.001 | 1,97 | 12.87 |
| **C. Total free amino acids** | **$R^2$** | ***P*** | **DF** | **F** |
| FAA = 68.1 + (4.9 × TIN) | 0.39 | <0.001 | 1,97 | 61.28 |
| FAA = 136.0 - (3.1 × Light) | 0.18 | <0.001 | 1,97 | 21.42 |
| FAA = 171.5 - (4.7 × Temp) | 0.15 | <0.001 | 1,97 | 16.93 |

### 4.4.6 Season and growth

Growth rates were compared for all sites examined in winter 2003 and summer 2004. There was a clear overall difference in growth rates between seasons with rates in the summer being close to double those measured in the winter (Figure 4.16, A and B).

However, there was no clear difference in growth rates (based on increases in fresh weight biomass) between the sites in either the summer or the winter. In addition, growth did not correlate with any tissue-N indices measured (data not shown). However, it was noted that growth in the summer was very vigorous resulting in very

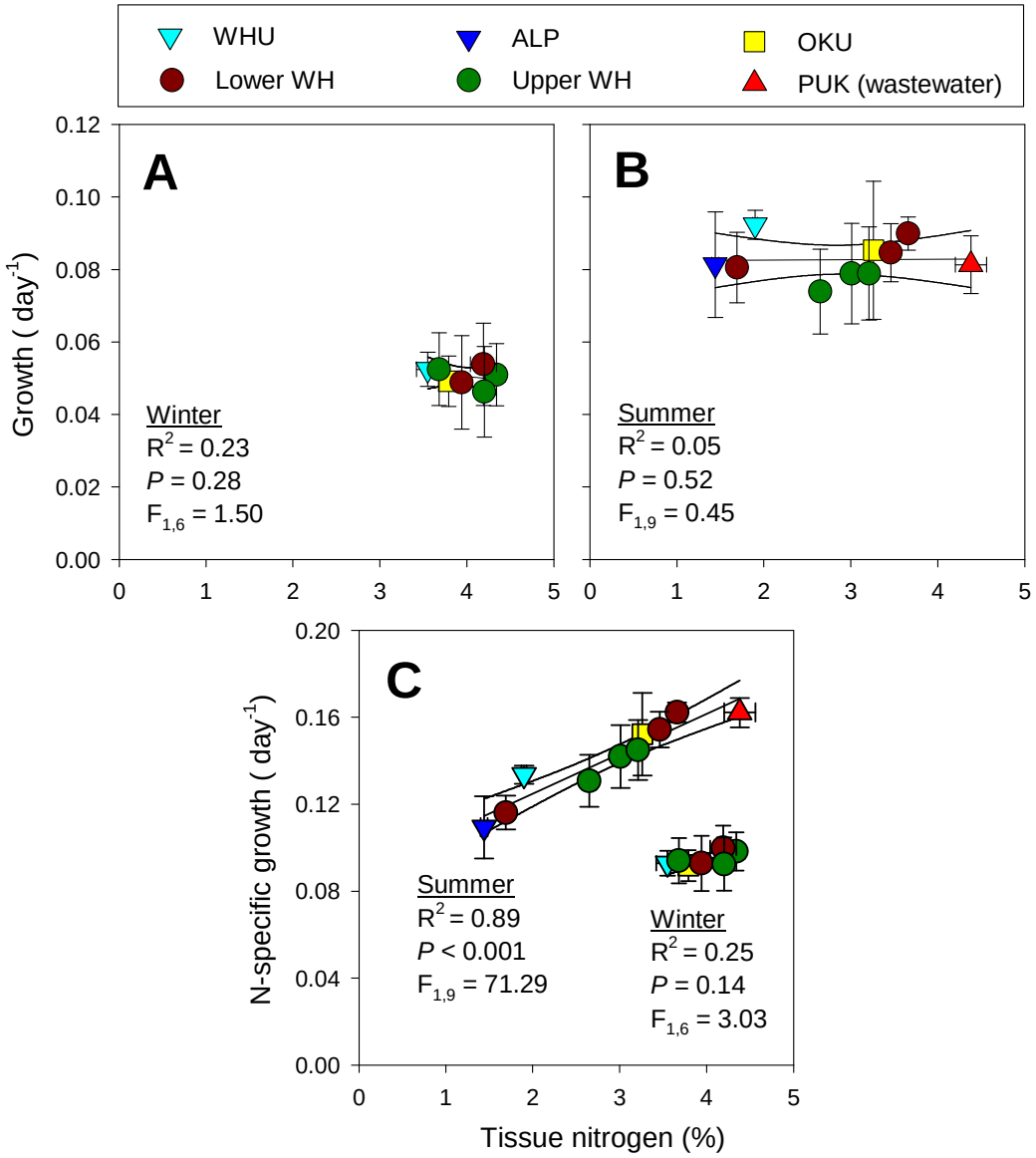



**Figure 4.15.** Comparison of change in growth rate with change in total nitrogen content in test-*Ulva* from seven sites in (**A**) winter (2003) and ten sites in (**B**) summer (2004). Change in (**C**) N-specific growth with change in total nitrogen content in winter (2003) and summer (2004). Values are means ± standard errors for 3 replicate stations.

full enclosures which may have inhibited further growth. When specific growth was expressed as N-specific growth there was strong correlation ($R^2$ = 0.89) with TN in test-*Ulva* in the summer data but not winter (Figure 4.16, C).

### 4.4.7 Nitrogen isotopes

Patterns in N-isotopes from test-*Ulva* showed very little seasonal variability within sites, although *Ulva* from all sites except ALP had values that were significantly different from that at the WHU reference site (Figure 4.16). Values ranged from 7.2 ‰ in *Ulva* at ALP to 14.7 ‰ in *Ulva* at PUK. The PUK site was not only the most enriched site, but since it was known to be affected by tertiary-treated sewage was almost certainly enriched in denitrified nitrogen. The lowest values were recorded at both WHU and ALP, and ranged between 7.6 ‰ and 8.5 ‰. For all WH sites $\delta^{15}N$ values were higher than those at the Whangateau Harbour (WHU) reference site (Figure 4.17). It was also apparent that all the Upper WH sites (and the WAU) sites were very similar to each other throughout the year. The Okura site (OKU) which was less likely to have been influenced by anthropogenic nitrogen compared with the WH sites, also had high $\delta^{15}N$ values. All of these sites except the WHU and ALP

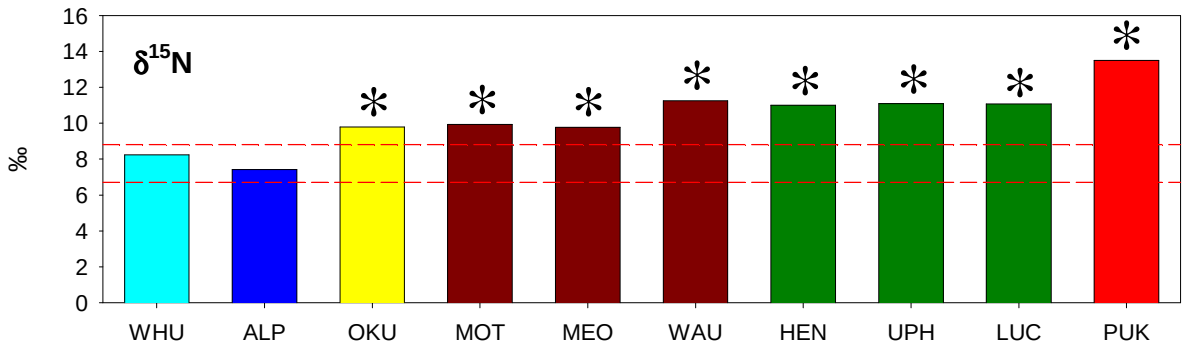



**Figure 4.16.** Pooled seasonal $\delta^{15}N$ isotope values in test-*Ulva* deployed at surface moorings at ten sites around Auckland during 2003/2004 (Note that for ALP, WAU and PUK samples were collected in summer 2003/2004 only). One-way Repeated Measures ANOVA: $F$ = 39.7, $P$ < 0.001. Asterisks indicate those sites that differed significantly ($P$ < 0.05) from the control site (WHU) according to the Holm-Sidak procedure for multiple comparisons versus a control. Values are means for four seasons ± standard error of the means. Note that the dashed lines represent the range of values found in *Ulva* growing on rural exposed coasts around New Zealand (Chapter Two).

sites fell outside the natural seawater isotope range identified in Chapter Three for exposed sites around New Zealand (Figure 4.17).

### 4.4.8 Heavy metals

Metal content in test-*Ulva* tissue from the 10 summer sites was examined. The following 8 metals recorded in test-*Ulva* were ranked in order from highest to lowest: Al > Fe > Mn > Zn > Cu > Pb > Ni > Cr (Figure 4.18). The greatest range in values for metal content were shown by Al, Fe, Pb and Cr, with the lowest values nearly always recorded in test-*Ulva* at the two reference sites WHU and ALP (Figure 4.18). The metals that showed the least dramatic range were Zn, Cu and Ni. However, even for these metals the amount contained in *Ulva* tissues at the two reference sites was low compared to that in most other sites. Test-*Ulva* at PUK had some of the highest tissue metal concentrations while metal concentrations at OKU were similar to, or sometimes higher, than the WH sites. Test-*Ulva* at LUC recorded the highest tissue concentrations of Zn and Cu.

There were generally higher levels of both Al and Fe in all urban environments and at OKU. Al and Fe were both accumulated to concentrations close to two orders of magnitude more than all other metals analysed (excluding Ca, K, Mg and Na). It was also noted that the sites with higher concentrations of Al and Fe also tended to have higher levels of fine sediments present. Although no measures of turbidity were made previous studies have shown that sediment levels in the Hauraki Gulf tend to increase closer to Auckland (Paul, 1968; Grace, 1983). Finally, there were strong correlations between Al, Fe, Mn, Ni and Cr, and between most metals and tissue N content (Table 4.3).

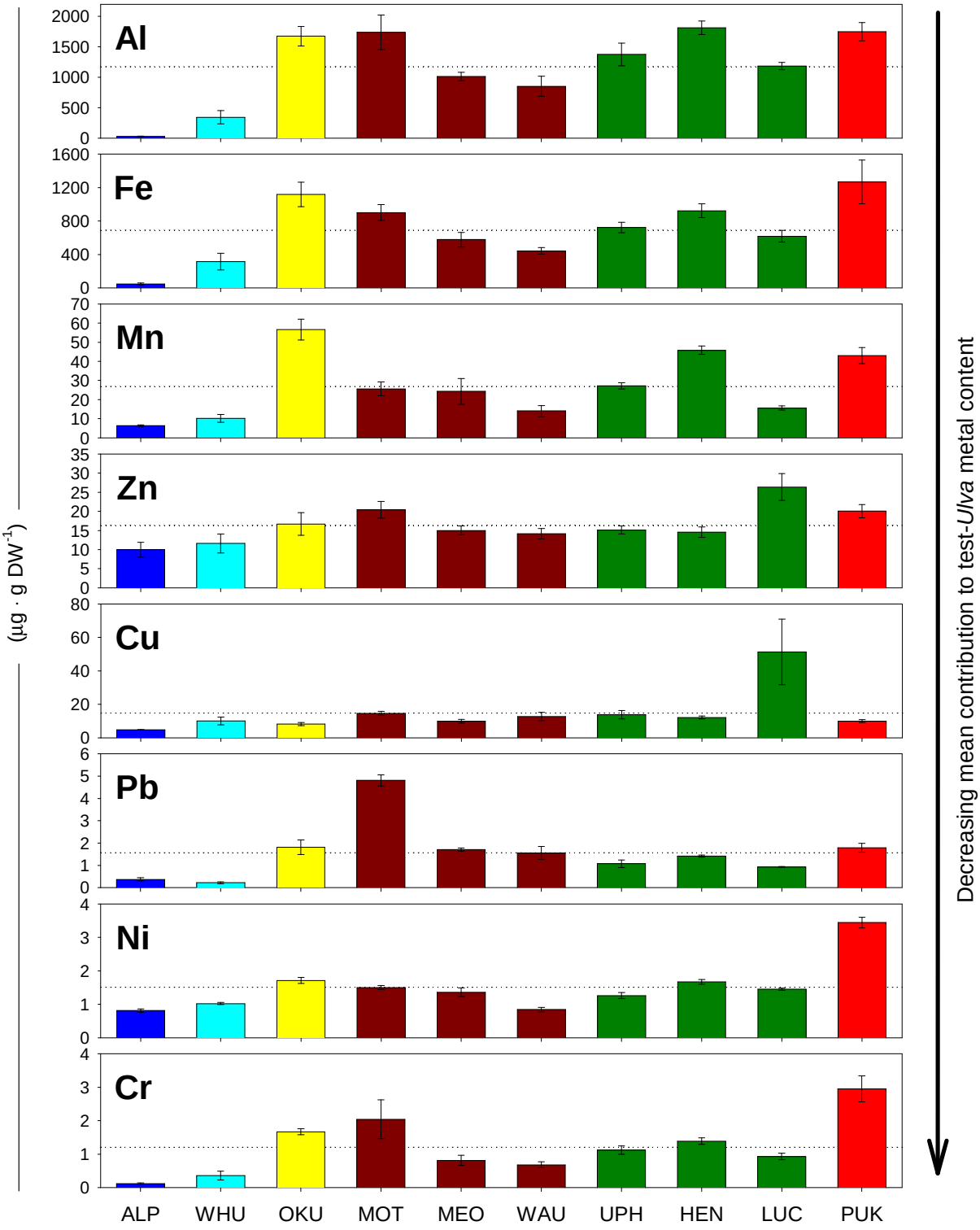



**Figure 4.17.** Comparison of metal content in test-*Ulva* tissue at 10 sites in the Auckland region in February 2004. Values are means ± standard errors for 3 replicate stations. Overall mean tissue value for each metal indicted as a dotted line.

**Table 4.4.** Spearman correlation matrix for tissue metal content and tissue nitrogen in *Ulva* deployed at 10 sites in the Auckland region in February 2004.

| | Al | Mn | Zn | Cu | Pb | Ni | Cr | Tissue-N |
|---|---|---|---|---|---|---|---|---|
| **Fe** | **0.939 ***** | **0.939 ***** | 0.673 * | 0.103 | 0.697 * | **0.952 ***** | **0.964 ***** | 0.758 ** |
| **Al** | | **0.891 ***** | 0.612 | 0.261 | 0.624 * | **0.891 ***** | **0.927 ***** | 0.697 * |
| **Mn** | | | 0.515 | 0.006 | 0.648 * | **0.867 ***** | **0.855 ***** | 0.624 * |
| **Zn** | | | | 0.527 | 0.552 | 0.697 * | 0.758 ** | 0.782 ** |
| **Cu** | | | | | -0.006 | 0.042 | 0.224 | 0.103 |
| **Pb** | | | | | | 0.661 * | 0.77 ** | 0.758 ** |
| **Ni** | | | | | | | **0.915 ***** | **0.842 ***** |
| **Cr** | | | | | | | | **0.830 ***** |

*P*-values indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bold font indicates highly significant correlations.

## 4.5 Discussion

### Ulva *as a biological integrator of nitrogen loading*

From this work it became clear that although seawater TIN concentrations were reflected in N-indices from test-*Ulva*, these algae were probably integrating available seawater nitrogen over time scales that were poorly represented by values measured in most instantaneous seawater samples (e.g., Figure 4.11). Test-*Ulva* chlorophyll, and therefore tissue nitrogen, integrated differences between environments over a period of weeks (Figure 4.7). However, Experiment 3 also demonstrated that N-indices in test-*Ulva* were capable of integrating the average nitrogen concentrations at tidal scale pulses (Figure 4.14). From previous work (Chapter Three, Figure 3.5, A) free amino acids in test-*Ulva* were more likely to change quickly over the short (possibly days) average concentrations and therefore were more likely to reflect short term changes in seawater TIN concentrations. This was supported by an overall correspondence between FAA and measured seawater TIN (Figure 4.10). However, given the differences in the regression relationships from the seven sites followed through the year (Figure 4.11), it is probable that other factors were involved in the way that biochemical changes in test-*Ulva* related to measurements of seawater nitrogen. Tidal aliasing of seawater samples was one probable factor, but it is also possible that test-*Ulva* were 'seeing' more nitrogen than was measured (e.g., organic nitrogen sources).

In terms of relative differences between sites, FAA N-indices provided the clearest and most consistent contrasts between the low nitrogen reference site (WHU) and sites like MOT in the Waitemata Harbour. OKU was intermediate and it was highly probable that ammonium recorded in seawater samples at low tide were not completely integrated in N-indices in test-*Ulva* over tidal average time scales. OKU was a very sediment impacted site compared with WHU. The high ammonium levels at OKU were possibly derived from efflux from sediments exposed at low tide (as reported by Hou *et al.* (2005)), although the high levels may equally have been due to unnatural sources.

***Seasonal effects on Test-*Ulva *tissue N-indices***

In addition to the issues of seawater nutrient variability another potential difficulty highlighted in this study was the confounding effects of season. Seasonal fluctuations in N content are well documented for macroalgae, including *Ulva* (Chapman and Craigie, 1977; Rosenberg and Ramus, 1982; Wheeler and Weidner, 1983; Thomas and Harrison, 1985), and can be strongly influenced by either temperature (Rosenberg and Ramus, 1982; Duke *et al.*, 1987; Duke *et al.*, 1989a; Altamirano *et al.*, 2000) or light (Duke *et al.*, 1987; Duke *et al.*, 1989a; Coutinho and Zingmark, 1993; Altamirano *et al.*, 2000). There was a clear seasonal increase in N-indices in the winter. This was particularly apparent in increases in TChl and TN at the low-N reference site at WHU. However, all sites also had increases in seawater TIN concentrations in winter to varying degrees. Since these increases were also coincident with decreases in irradiance and temperature in the winter it is possible that elevated seawater TIN concentrations and reduced seawater temperature and/or irradiance had an additive effect resulting in consistently higher TN and TChl levels in test-*Ulva*. However, statistical analysis (Table 4.2) suggested that seawater TIN concentration had the strongest influence on N-indices in test-*Ulva*.

Growth rates in the winter were slightly over half those obtained in the summer. In neither season was fresh-weight growth correlated with seawater nitrogen concentration nor tissue-N content (Figure 4.15) despite a large range of TN values (1.4 to 4.3 %) observed in test-*Ulva* from the different sites. Some laboratory studies examining nitrogen-limited growth in *Ulva* suggest critical-N values below about 2 % (Björnsäter and Wheeler, 1990; Pedersen and Borum, 1997). However, the lowest TN

value in test-*Ulva* was 1.4 % recorded at ALP in the summer, but it would appear from the data presented here that growth was probably not N-limited in any situation. This may have related to the fact that test-*Ulva* were deployed on the surface and would therefore have seen constant water motion (either from surface driven wind waves or ripples and/or tidal currents). In addition, the seasonal shift in TN content in test-*Ulva* from the low-N WHU reference site (increasing from 1.9 % in the summer to 3.6 % in the winter) was greater than that seen in sheltered low-N sites in Chapter Two (see Figure 2.9, A). Therefore, it is possible that surface moored sites functioned more like the exposed sites in natural populations (see Figure 2.9, B). Irrespective of the possible effects of water motion on N-status in test-*Ulva*, there were clearly other factors limiting *Ulva* growth that were associated with winter conditions. These were likely to have been temperature and/or irradiance. This is consistent with the previous suggestions (Chapter Two and Chapter Three) that reduced growth probably lead to an increase in TN (and TChl) because external nitrogen was in surplus supply for growth requirements in *Ulva*.

***Nitrogen isotopes***

The $\delta^{15}N$ values measured in test-*Ulva* in this study were used to provide qualitative information on the different environments only (as they were in Chapter Two) since source signatures were not measured. Except for sites that were considered to be of low impact (i.e., WHU and ALP) all sites resulted in test-*Ulva* with values that were outside (higher than) the range for clean seawater from exposed coastal sites (Chapter Two). On the other hand the similarity between the Upper Waitemata Harbour sites (HEN, UPH and LUC) presumably relates to the fact that it is a large harbour, with a large tidal prism and longer (9 – 11 tidal cycles (Auckland Regional Authority, 1983)) retention times. The fact that these similarities persisted over the year also suggests that the chemical processes controlling system $^{15}N$ fractionation were not seasonally affected. Since there was no significant denitrified (i.e., tertiary treated) sewage source to the Waitemata Harbour it is suggested that denitrification occurring in sediments (Cohen and Fong, 2006)) was a significant factor affecting ambient nitrogen isotopic composition in this harbour.

Although results from this survey suggest that higher $\delta^{15}N$ values provide qualitative information on nitrogen source they do not necessarily, in isolation, provide

information on the amount of nitrogen present in seawater. For example, the MOT and MEO sites were both identified as having relatively high nitrogen loading throughout this survey and yet test-*Ulva* at these two sites recorded lower $\delta^{15}N$ values compared to the other WH sites (WAU, UPH, LUC and HEN) (Figure 4.16). It is probable that the lower isotopic values in test-*Ulva* at MOT and MEO are the result of the mixing of nitrogen sources that were both heavy in $^{15}N$ isotopes (i.e. the same as that affecting the other WH sites) and light in $^{15}N$ isotopes (i.e., derived from either a local point source, presumably from one or both of the adjacent Motions and Meola creeks, or from intrusions of the isotopically lighter source water from outside the harbour). From this study it would appear that higher $\delta^{15}N$ values were generally associated with urban impacted environments. The OKU site being predominantly rural also had consistently higher $\delta^{15}N$ values (relative to the WHU reference site). It is possible that this was due to either an unnatural nitrogen source (e.g. that derived from septic tanks) or more probably that suggested for other sediment impacted environments above.

***Heavy metals***

In terms of the composition and the range of values of metals bound in test-*Ulva* tissues, data in the current study were comparable with similar studies on *Ulva* species (Haritonidis and Malea, 1999; Villares *et al.*, 2001; Gosavi *et al.*, 2004; Strezov and Nonova, 2005). Villares *et al* (2001) showed significant correlations between tissue levels of Al, Cu, Fe and Zn and the labile fraction in sediments. However, to what extent tissue metal content in test-*Ulva* reflected the dissolved fraction in seawater is unknown, but since test-*Ulva* used in this study was deployed at the surface further away from sediments it could be assumed that tissue metal levels were more likely to reflect the dissolved fraction in seawater. Al and Fe were both accumulated to concentrations close to two orders of magnitude more than all other metals analysed (excluding Ca, K, Mg and Na).

There were strong correlations between Al, Fe, Mn, Ni and Cr, and also between most metals and tissue N content (Table 2). Moreover, it is known that there are interactions between some metals and nitrogen content in *Ulva* tissues (Munda and Veber, 1996; Lee and Wang, 2001; Munda and Veber, 2004). This partly relates to the fact that several metals act as cofactors of metalloenzymes (e.g. Fe, Mn, Zn, Cu

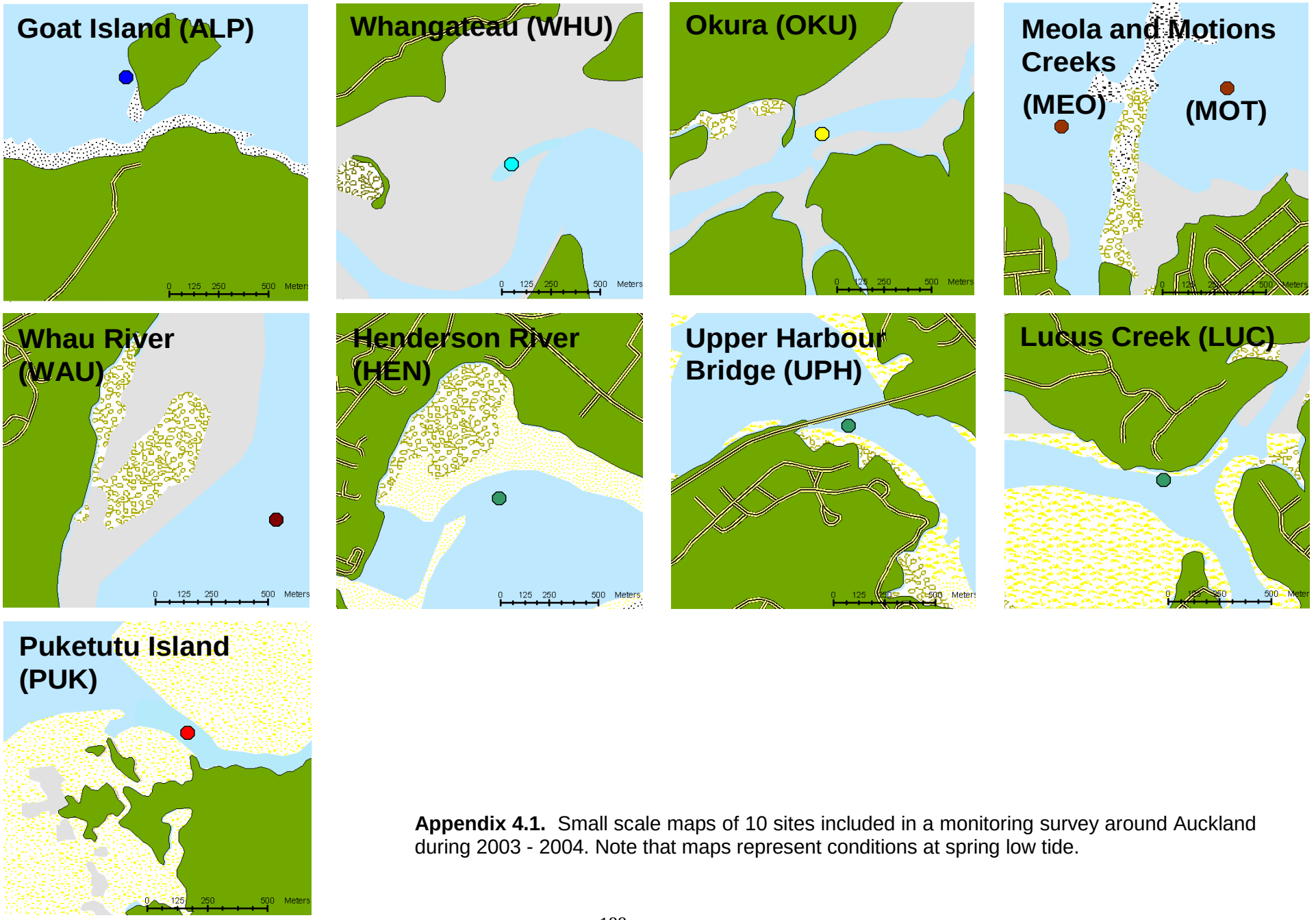
and Co) and are required for growth in seaweeds. This has potential implications for interpretation and suggests that some metal levels recorded in *Ulva* tissues may be the result of such interactions since *Ulva* with a higher N-content would also presumably contain more protein and therefore more sites for bound cofactor metals. On the other hand this doesn't discount the possibility that differences were due to the chemical environment. It is possible that the same point or non-point sources that lead to elevated nitrogen concentrations (as reflected in *Ulva* tissues) might also simply contain higher concentrations of dissolved metals as well. However, not all metals correlated well with other constituents in *Ulva* tissues in this study (Table 4.3). One such metal was Pb, which showed a particularly high peak at MOT, but not at the nearby MEO site (Figure 12). In a study of *Ulva* as an indicator of heavy metals it was shown that Pb was particularly well correlated with dissolved seawater concentrations of this metal (Malea and Haritonidis, 2000).

***Conclusions***

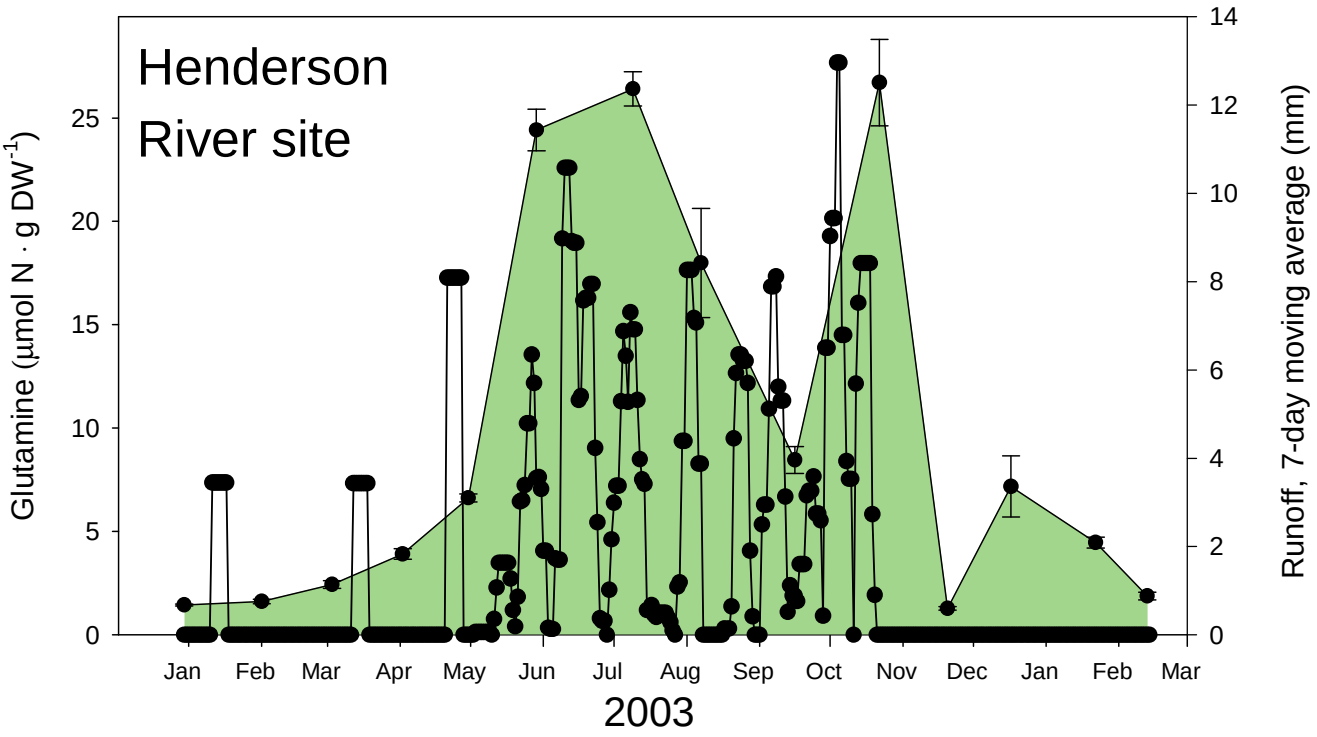
The objective of this chapter was to develop a practical seaweed indicator that was robust to experimental manipulation, could be deployed into a range of different environments and then after time could provide useful information about these environments. The objective was not necessarily to provide information on the causal relationships between environment and indices in *Ulva*, but to demonstrate that *Ulva* used in this fashion, was fundamentally robust (i.e., it survived and grew) and reflected differences between a range of different environments. In the process of investigating the utility of *Ulva* for this purpose, this study has also highlighted some of the inherent difficulties with monitoring seawater column nutrients using conventional measurements of instantaneous seawater samples. For example, test-*Ulva* consistently showed biologically relevant differences in nitrogen loading between three environments (WHU, OKU and MOT) that were not reliably detected by conventional seawater monitoring approaches. Moreover, in the harbour and estuarine environments investigated in this study, representing a range of very different mixing zones (of 'new' seawater and nutrients derived from terrigenous sources), test-*Ulva* (of identical initial low-N status prior to deployment) consistently demonstrated differences in levels of biologically integrated nitrogen loading. Using conventional approaches this may only have been achievable with expensive *in situ*

autoanalyser technology measuring nutrient concentrations over tidal cycles for prolonged periods of time.

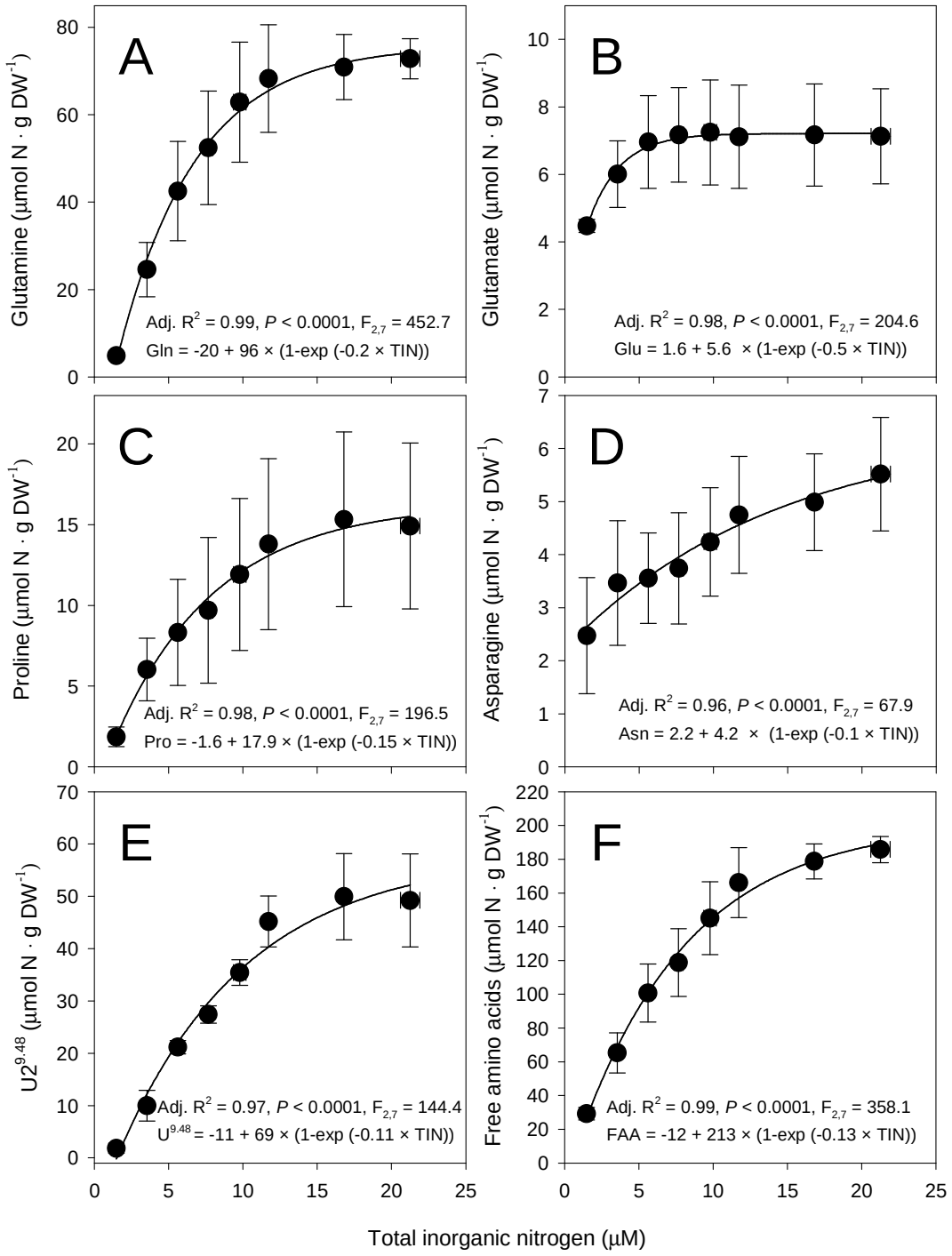
As suggested in Chapter Two the combination of quantitative (e.g. FAA content) and qualitative (isotopic composition) information from seaweed indicators, such as *Ulva*, would appear to provide useful information for water quality management. However, I suggest that there is also an opportunity to broaden the definition of biological indicator to that of biological integrator; an organism that integrates the average value of an environmental variable prone to fluctuation.




**Appendix 4.1.** Small scale maps of 10 sites included in a monitoring survey around Auckland during 2003 - 2004. Note that maps represent conditions at spring low tide.




**Appendix 4.2.** Comparison of values of freshwater runoff with change in glutamine levels in test-Ulva from adjacent to the Henderson River.




**Appendix 4.3.** Change in levels of (**A - E**) selected individual amino acids and (**F**) total free amino acid pool in test-*Ulva* with change in inorganic nitrogen concentration.. Regression curves are fitted using SigmaStat 9 with an exponential rise to maximum model. Values are means ± standard errors for 3 separate experiments.

# Chapter Five
# General discussion

***Are differences in* Ulva *tissue-N indices due to environment or taxonomy?***

The survey of natural *Ulva* populations (Chapter Two), which probably included several species, showed a large range of nitrogen status (0.7 to 4.5 %). The question therefore arose; to what extent might taxonomic or environmental differences account for observed patterns in tissue-N indices in *Ulva*? In one special case where two distinct morphologies of *Ulva* were found growing side-by-side in two environments contrasting in nitrogen loading, differences in N-indices (and $\delta^{15}N$) between the two sites strongly suggested that it was environmental rather than taxonomic differences that controlled N-content in *Ulva* species (Chapter Two, Figure 2.11). From other research in has been shown that *Ulva curvata* and *Codium decorticatum*, exhibit seasonal differences in nitrogen allocation that are largely due to temperature (and probably light), and are apparently independent of morphological and genetic differences between species (Duke *et al.*, 1987). This was clearly consistent with the observed differences between *Ulva* collected in the summer and winter from around New Zealand (Chapter Two).

From this research it was proposed that, relative to growth rate and the effects of season, differences in nitrogen availability should have the predominant effect on the biochemistry of nitrogen metabolism in *Ulva*, compared with those relating to taxonomy or morphology. However, there were also noticeable differences between the relative composition of some N-indices in natural populations of *Ulva*. One was the ratio of chlorophyll *a* : *b* which suggested that differences between species may be due to taxonomy or morphology (i.e., *U. fasciata* had a distinctly different ratio compared with the other three *Ulva* species, Figure 2.11). This is consistent with at least one study which concluded that values of chlorophyll *a* : *b* in different seaweeds, including several *Caulerpa* species and *U. lactuca*, were largely species-

specific (Keast and Grant 1976). Another difference between the *U.* (syn. *Enteromorpha*) *intestinalis* and the two other frondose *Ulva* species was the ratio of total chlorophyll : tissue nitrogen. Previous examinations of natural populations of *U. intestinalis* have shown a linear relationship between total chlorophyll (chlorophyll *a* + *b*) and tissue nitrogen with approximately 4.1 mg · g $DW^{-1}$ chlorophyll for every 1 % of tissue nitrogen (Barr and Rees, 2003). However, most *Ulva* in the current study contained on average 2 mg · g $DW^{-1}$ total chlorophyll (chlorophyll *a* + *b*) for every 1 % of tissue nitrogen. Direct comparison of values from this survey with that from the study of Barr & Rees (2003) suggest strong differences between total chlorophyll : total tissue-N ratios in *Ulva intestinalis* and most of the mixed frondose *Ulva* species in this survey (Figure 5.1).

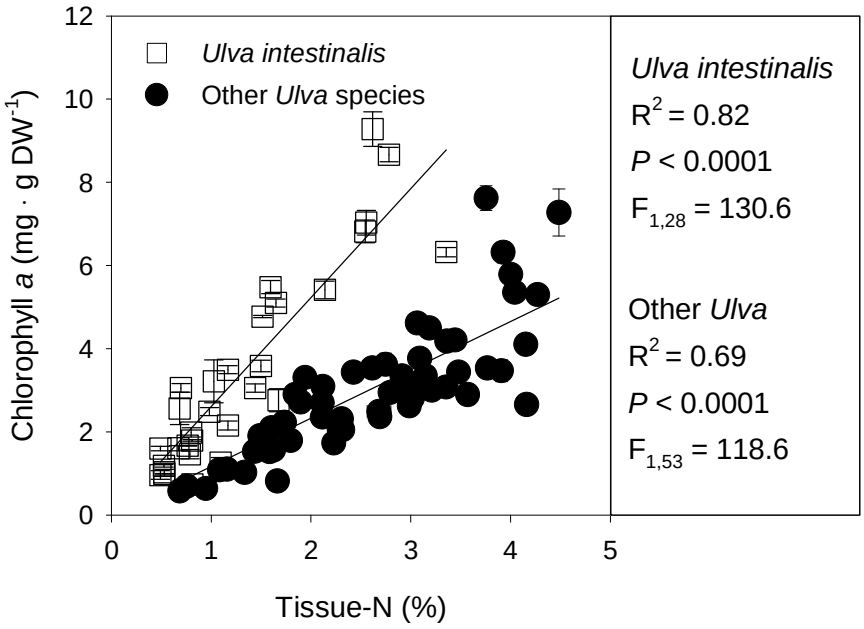



**Figure 5.1.** Comparison of changes in total tissue-N with changes in chlorophyll *a* between morphologically distinct *Ulva intestinalis* (unfilled squares) from the study of Barr & Rees (2003) with that from mixed *Ulva* species (filled circles) from the *Ulva* collected around New Zealand in summer and winter 2003. Values are means ± standard errors for three replicate samples.

In addition to the differences in relative chlorophyll composition, this research has also shown that some amino acids may be accumulated differentially in response to

nitrogen addition. Most natural *Ulva* could produce high concentrations of asparagine under conditions of high seawater nitrogen, or when seasonal or other physical factors (i.e., low temperature and light, or exposure to greater water motion) resulted in nitrogen saturation in *Ulva* tissues. However, it was also known that not all *Ulva* accumulated high concentrations of asparagine. For example, when enriched with 10 μM ammonium in outdoor cultures there was no significant increase in asparagine levels in either *U. spathulata* from rock pools or *U. pertusa* from the Mokohinau Islands (MK) (unpublished data). In contrast, *U. pertusa* from Otumoetai (OT) maintained under identical conditions at the same time as *U. pertusa* from the Mokohinau Islands produced increased levels of asparagine, suggesting that the control of asparagine synthesis was more complex than *Ulva* taxonomy would suggest (unpublished data). Chapter Four focused entirely on an *Ulva* species that never produced asparagine in high concentrations, even under artificial nitrogen enrichment. This *Ulva* did however, produce much greater levels of glutamine in response to nitrogen loading than most other natural populations. This may have been due to either low levels or reduced gene expression of enzymes such as asparagine synthetase (AS), resulting in the accumulation of glutamine.

Irrespective of this seemingly fundamental difference in free amino acid composition between the two chemomorphs of *Ulva* examined in this study, it appeared to make little difference to the overall responses of the total FAA pool to external nitrogen concentration. The reason for this might relate to the fact that most amino acids respond, at least to some degree, to changes in external nitrogen concentration (as shown in Chapter Three, Table 3.3). It is recognised that amino acid analysis by HPLC is time consuming and relatively expensive. Therefore, given that the total FAA pool responded to changes in external nitrogen concentration in a similar way to glutamine and asparagine (because they were dominant amino acids), a simple ninhydrin assay for total amino acids (or fluorescence as used by Naldi and Wheeler, 1999) may provide a simple, cheap and useful indicator of nitrogen loading.

**Ulva *as an integrator of nitrogen loading and the nitrogen isotopic source pool***

*Ulva* was chosen as a study species because it is a relatively simple, undifferentiated alga that grows rapidly, has a high surface area : volume ratio (Taylor *et al.*, 1998), and as a corollary high rates of nitrogen (ammonium) uptake (Pedersen, 1994; Taylor

*et al.*, 1998). *Ulva* can be defined as a simple *R*-strategist, without complex chemical defences or storage compounds. As a result of these attributes *Ulva* is likely to reflect relatively short term changes in the environment in compared with slower growing macroalgae. It is ubiquitous, and is therefore available to be sampled in a wide variety of environments. Moreover, it is robust to experimental manipulation and relatively easy to grow in culture over prolonged periods, and was therefore an appropriate choice as a standardised test-organism in the field.

To test whether N-indices in *Ulva* reliably reflect nitrogen loading in the environment, both natural and artificial populations of *Ulva* were examined in relation to seawater nitrogen concentration. However, during the course of this research it became apparent that nitrogen concentrations measured in instantaneous seawater samples were probably poor predictors of the nitrogen available to *Ulva* on a time average basis (e.g., Experiment 1, Chapter Four). Conventional measurements of water column nutrients do not necessarily give good information on what is actually biologically available to primary producers (Fong *et al.*, 1998), particularly in environments that are prone to fluctuations in nutrient concentrations (e.g., as a result of tidal fluxes). Similarly, conventional seawater measurements of an element (e.g., heavy metals) are not equivalent to its bioavailability (Brian *et al.*, 1985; Villares *et al.*, 2001).

It is generally accepted that macroalgae can integrate information on concentrations of nutrients, because they store that which is in excess of growth requirements (Hanisak, 1979; Björnsäter and Wheeler, 1990; Fong *et al.*, 1994). The more surplus nitrogen there is the more N-saturated tissues become (assuming constant growth rate). However, until relatively recently, total tissue-N content (TN) or the ratio of nitrogen : carbon (N : C) was the only reliable indicator of N-content in common use in studies of microalgae (Flynn *et al.*, 1989) and macroalgae (Atkinson and Smith, 1983; Björnsäter and Wheeler, 1990; Al-Amoudi, 1994; Peckol *et al.*, 1994; Jones *et al.*, 1996; Fong *et al.*, 1998). More recent research has examined the responses of free amino acids in macroalgae to nitrogen loading, with positive results (Horrocks *et al.*, 1995; Jones *et al.*, 1996; Barr, 2000; Costanzo *et al.*, 2000; Barr and Rees, 2003). Accordingly, the current research has further investigated the biochemical constituents (N-indices) in *Ulva* which work at opposites ends of the temporal N-

integration spectrum (i.e., short term changes subsequent to assimilation and longer term, damped changes in total tissue nitrogen).

The primary aim was to determine what N-indices in *Ulva* would most reliably reflect the presence of high concentrations of nitrogen in seawater. From previous research (e.g., Fong *et al*., 1998; Jones *et al*., 1996), and results in the current study, it has been shown that total tissue nitrogen and chlorophyll content are indicators of nitrogen availability in seawater. However, from the current research it is suggested that the free amino acid pool and its individual constituents provide a higher resolution biological proxy for nitrogen loading in seawater. In the current study the FAA pool represented 3 - 14 % of nitrogen present in *Ulva* tissues. It is suggested that the FAA pool better resolves levels of biological nitrogen loading, largely as a consequence of its transitory nature and possibly its diminutive size (i.e., a small change in N taken up is more likely to be detected in a small pool compared to being detected as a small percentage change relative to the total pool of nitrogen). However, virtually nothing is known about how free amino acid and total nitrogen in macroalgae differentially integrate nitrogen availability with respect to time.

From the first experiment in Chapter Three (Figure 3.4) it was clear that chlorophyll could take considerable time (up to three weeks) to change with respect to nitrogen addition. Moreover, field deployment of test-*Ulva* supported this conclusion with equilibration of chlorophyll levels (and therefore tissue-N) to seawater at three different sites, occurring over a similar period of time (Figure 4.7). At the end of this experiment all N-indices, particularly the amino acids, indicated differences between these environments that were not necessarily discernable by conventional water quality monitoring. However, while it was not specifically determined how fast the amino acid pool changed in response to changes in seawater nitrogen availability, it can be said that the FAA pool was capable of integrating the average of a pulsed supply of nitrogen over tidal cycle time-scales (Figure 4.14). Further research is required to ascertain how the FAA pool (or its constituents) responds to different nutrient pulse regimes (e.g., period and asymmetry of pulse). In addition, there will be other factors, biotic and abiotic alike, that will impinge on how average nitrogen availability in seawater will be reflected in the biochemistry of *Ulva* tissues.

However, by using a standardised test-organism, interpretation of its metabolic responses is largely limited to the effects of the environment in which it grows.

Nitrogen isotope values in *Ulva* were clearly a powerful indicator of differences in the isotope source pool in seawater. This was inferred from the similarity of signatures in open coastal *Ulva*, and from the relatively small fractionation in *Ulva* supplied with contrasting N-source, under both light-limited and light-saturated conditions. These conclusions are supported by other experimental work on *Ulva* (sym. *Enteromorpha*) *intestinalis* in response to isotopic source and concentration (Cohen and Fong, 2005). While $\delta^{15}N$ signatures in macroalgae are clearly useful tracers in studies of the effects of sewage nitrogen, for example, studies involving them usually require additional isotopic information about the potential sources for use in mixing models. However, measuring the source signature in seawater can be analytically challenging and expensive. It is suggested that if *Ulva* does closely reflect the source pool (perhaps better than other macroalgae) it may be a useful biological proxy for this parameter. In a food web study by Vizzini and Mazzola (2006) in oligotrophic waters in the Mediterranean, it was concluded that spatial and temporal isotopic (in $\delta^{15}N$ and $\delta^{13}C$) heterogeneity within primary producers, and primary and secondary consumers, was largely an attribute of the environment. However, within the group of primary producers examined, *Enteromorpha* sp. showed little spatial (between two similar sites) or temporal (across four seasons) variability in $\delta^{15}N$ at values close to 12.7 ‰.

In the case of New Zealand $\delta^{15}N$ values in *Ulva* outside the range of 6.7 to 8.8 ‰ (being similar to that suggested by Rogers, 1999 and Rogers 2006, see Chapter Two) may be useful for indicating the presence of unnatural nitrogen, or at least nitrogen that has undergone chemical transformation (e.g. in sediments). Moreover, it is also suggested that the combination of $\delta^{15}N$ values (as a qualitative N-index) and quantitative N-indices (such as the FAA pool) in *Ulva* tissue has the potential to provide useful information about both the amount and composition of nitrogen entering coastal environments. However, it would appear that quantitative indices may more reliably indicate contrasting differences in nitrogen availability in the summer (since N-status appears to be more affected by factors other than nitrogen

availability in the winter conditions) when increased nitrogen loading is likely to cause increases in peak seasonal growth.

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