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Uptake and transport of nitrogen derived from sessile epifauna in the giant kelp *Macrocystis pyrifera*

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ABSTRACT: Sessile epifauna that excrete ammonium as a waste product are often closely associated with surfaces of macroalgae that are able to absorb and use ammonium for growth. This close association means that nitrogen provisioning from epifauna to host macroalgae is probable, but barriers to uptake, formed by epifaunal colonies, and the physiological status of colonised macroalgal tissue may limit the amount of ammonium absorbed by colonised macroalgae. Evidence for the movement of nitrogen from sessile epifauna into and around the giant kelp *Macrocystis pyrifera* was gathered using a ^{15}N-enriched stable isotope tracer. Experiments in which ^{15}N-labelled phytoplankton was fed to the epifaunal bryozoan *Membranipora membranacea* did not to find conclusive evidence for the flow of nitrogen released by bryozoan colonies into colonised *M. pyrifera* tissue. The low degree of transmission of ^{15}N observed may be due to the barrier to nitrogen uptake that is formed by *M. membranacea* tissue and the low affinity for ammonium uptake by *M. pyrifera* blades during winter. *M. membranacea* is unlikely to provide significant benefits to host macroalgae via nitrogen provision in many situations, due to its sheet-forming habit and its absence during summer low-nitrogen periods. *In situ* incubations of *M. pyrifera* blades in ^{15}N-enriched ammonium were used to determine if ammonium provided by epifaunal colonies could be taken up by mature blades tissue and exported to the stipe for long-distance transport to actively growing meristems. Uptake and export of labelled nitrogen from basal blades into the stipe was measured, and the potential for long-distance transport of nitrogen from older blades was confirmed. Active transport of nitrogen from older macroalgal tissue colonised by epifauna such as hydroids could be an important mechanism allowing kelp with specialised long-distance solute transport systems to tolerate nitrogen limitation.

KEY WORDS: Ammonium excretion · Bryozoans · Epifauna · Kelp · Long-distance transport · *Membranipora membranacea* · *Macrocystis pyrifera* · Nitrogen · Stable isotope tracers · ^{15}N

INTRODUCTION

Nitrogen is the major limiting nutrient for macroalgal primary productivity (Hanisak 1983) and therefore is an important factor in the functioning of coastal ecosystems that rely on this productivity (Duggins et al. 1989). Mobile and sessile fauna that live on macroalgal surfaces may reduce the effects of nitrogen limitation via their excretion of ammonium (Gerard & Mann 1979, Probyn & Chapman 1983, Hurd et al. 1994, Taylor & Rees 1998, Hepburn & Hurd 2005, Fram et al. 2008). The cheilostomate bryozoan *Membranipora membranacea* is a key epifaunal species on macroalgal surfaces in both northern and southern hemispheres (Dixon et al. 1981, Seed & O'Conner 1981, Harvell 1986, Berman et al.

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1992, Lambert et al. 1992, Schwaninger 1999, Hepburn & Hurd 2005, Saunders & Metaxas 2008). Through the capture and digestion of food items (e.g. phytoplankton, detritus, dissolved organic matter; Ryland 1970, Winston 1977, De Burgh & Fankboner 1978), suspension feeding by *M. membranacea* colonies may make nitrogen bound within particulate matter in the water column available to its kelp substratum in the form of ammonium.

Membranipora membranacea is able to absorb exudates from macroalgal surfaces as a supplement to food it captures through suspension feeding (De Burgh & Fankboner 1978, Manríquez & Cancino 1996). In turn, ammonium diffuses rapidly from *M. membranacea* due to its high solubility and low molecular size. It has been proposed that some waste ammonium from *M. membranacea* is excreted through its base, which is in direct contact with the seaweed surface (Hurd et al. 1994). This pathway has been proposed as a basis for a mutualistic relationship between this bryozoan and the giant kelp *Macrocystis pyrifera* (Hurd et al. 1994) and could provide enhanced productivity of important kelp forest habitats over the wide geographic distribution of both partners (e.g. Schwaninger 1999, Steneck et al. 2002).

The hypothesis that *Membranipora membranacea* provides nitrogen to underlying *Macrocystis pyrifera* blade tissue was founded on the observation that *M. pyrifera* blade tissue beneath bryozoan colonies had higher nitrogen content when compared to surrounding bryozoan-free blade tissue (Hurd et al. 1994). However, flow of nitrogen from *M. membranacea* into colonised blade tissue has never been demonstrated. Feeding experiments utilising phytoplankton enriched with the stable isotope ^{15}N as food items were designed to determine if significant amounts of ammonium released by *M. membranacea* colonies during the feeding process (e.g. rupture of cells) and the production of waste products following digestion were taken up by underlying *M. pyrifera* blade tissue. The addition of ^{15}N labelled phytoplankton was predicted to enrich *M. pyrifera* blade tissue colonised by *M. membranacea* with ^{15}N but not bryozoan-free blade tissue as the process of feeding and digestion of phytoplankton by the bryozoan colony would be required to release labelled nitrogen stored within phytoplankton cells to make it available for uptake by blade tissue.

This study also aimed to identify if nitrogen excreted by sessile epifauna, such as bryozoans and other important epifaunal groups (e.g. hydroids) that are most common at the base of *Macrocystis pyrifera* fronds (Hepburn & Hurd 2005) can be made available to distant meristems at frond apices. Growth and nitrogen status of apical sections of *M. pyrifera* fronds have been demonstrated to increase with increasing cover of hydroids found on basal blades that were many metres distant from growing apices (Hepburn & Hurd 2005). Transport of nitrogen derived from epifauna growing on basal blades to distant meristems is possible through specialised cells found in the stipes of kelp (Raven 2003). These cells resemble the sieve tube elements of higher plants, and are able to transport carbon, mainly as mannitol, and probably nitrogen (as amino acids and protein) rapidly along the frond (Parker 1963, 1965, Lobban 1978, Schmitz & Srivastava 1979a, Manley 1983). Due to environmental (Gerard 1984) and physiological reasons (Kain 1982) it is estimated that the majority of *M. pyrifera* production occurs in the canopy region (Towle & Pearse 1973, Gerard 1984) and that basal blades that are senescent have limited roles in light harvesting. It is possible that basal blades that are heavily colonised by sessile epifauna could have a significant role in providing nitrogen to the apical and basal meristems of *M. pyrifera* if nitrogen uptake and transport capacity remains in mature blade tissue, despite apparent senescence and disintegration (Hepburn & Hurd 2005).

MATERIALS AND METHODS

Collection of *Macrocystis pyrifera* blade tissue for feeding experiments took place at Harington Point at the entrance to Otago Harbour, South Island, New Zealand (45° 47′ 03.5″ S, 170° 43′ 22.7″ E). *In situ* nitrogen transport experiments were conducted at a kelp bed at Omimi Cove (45° 41′ 29.0″ S, 170° 37′ 0.0″ E), a sheltered bay 13 km northwest of Harington Point. This area was chosen due to the lack of strong tidal currents that would have made carrying out this experiment at Harington Point difficult.

Laboratory feeding experiments

Dunaliella tertiolecta was selected as food for *Membranipora membranacea* in feeding experiments as it is an excellent food source for bryozoan colonies (Winston 1977). *D. tertiolecta* (innoculant from Portobello Marine Laboratory, University of Otago) was cultured in 1 l Erlenmeyer flasks in a culture medium made up with seawater filtered using Whatman GF/C filters (1 µm) and sterilized in an autoclave, and f/2 medium (Guillard & Ryther 1962) with 20 µM

10.4% ^{15}N-enriched $(NH_4)_2SO_4$ (Novachem) as the nitrogen source.

Culture greater than 3 wk old were used for feeding experiments as it was assumed that most ^{15}N-labelled ammonium would by then have been absorbed into phytoplankton biomass. Three litres of ^{15}N-enriched phytoplankton cultures were centrifuged in 500 ml centrifuge tubes at 1000 rpm for 5 min after which the supernatant was decanted off. The remaining phytoplankton mixture was centrifuged again for 5 min at 1000 rpm before the supernatant was decanted once more. This left 40 ml of highly concentrated phytoplankton for use in feeding experiments. Observations of phytoplankton through a microscope before and after centrifugation showed no evidence of a reduction in the numbers of motile phytoplankton as a result of this process. The concentrated phytoplankton solution was then placed in an incubator (Conviron Model E15) at 12°C for 2 h to acclimatize to experimental conditions.

Ten mature blades from the basal sections of separate *Macrocystis pyrifera* individuals that were colonised by *Membranipora membranacea* were haphazardly collected by SCUBA from 2 to 4 m depth at Harington Point during the austral winter on 6 July 2001, the day before experiments took place. The blades were covered in newspaper (that had been soaked in seawater) within covered bins for immediate transport to the laboratory 1 h away where they were placed in aerated seawater. To prevent damage to attached *M. membranacea* colonies, sections of bryozoan colonised blade tissue were cut from the blade around the colonies edge using a razor blade (bryozoan-colonised group). A second, identically shaped section of blade tissue was then cut from adjacent blade tissue (bryozoan-free group). Blade sections from the same blade remained paired for subsequent experiments. Blade sections were then placed in filtered seawater, in an incubator for 2 h at 12°C in the dark. Tissue discs were then placed in separate 100 ml 10% HCl-washed flasks containing 50 ml of low ammonium (< 1 µM) filtered seawater. Flasks were placed randomly on gently moving orbital shaker tables (Model SS70, Chiltern Scientific) in the incubator for 12 h at 12°C in the dark to facilitate acclimation by *M. membranacea* colonies and *M. pyrifera* blade tissue. An irradiance of 260 µmol m^{-2} s^{-1} was provided by 8 daylight fluorescent tubes (F72T12-CW-VHO, Sylvania) for 2 h before and throughout feeding experiments. Temperature and light levels were typical of those observed in shallow water habitats in southern New Zealand (Kregting et al. 2008, Hepburn et al. 2011).

Two treatment groups and 2 control groups of paired bryozoan-colonised and bryozoan-free blade tissue were used (n = 5). One ml of concentrated phytoplankton food was added to each treatment group, while no phytoplankton was added to control groups. All colonies were observed to be actively feeding during the incubation. Both control and treatment flasks were left in the incubator on orbital shaker tables set at 120 rpm for 8 h. Blade sections were then removed and washed in purified water. Bryozoan colonies were scraped off the blade tissue discs and dried in plastic tubes at 80°C. Blade sections were washed in purified water and all bryozoan tissue was removed before drying. Blade and bryozoan tissue were ground in a mortar and pestle before storage in sealed tubes prior to analysis for δ^{15}N. A separate mortar and pestle was used for the controls and ^{15}N-enriched blade and bryozoan tissue to prevent contamination.

In situ nitrogen transport in *Macrocystis pyrifera* fronds

To determine if ammonium, taken up by older blade tissue, could be exported from colonised blades and then transported along the stipe, ^{15}N-enriched nitrogen was traced from the site of uptake by blades near the base of fronds of *Macrocystis pyrifera* and along the stipe to apical and basal meristems *in situ*. Transport of ammonium-derived nitrogen in *M. pyrifera* fronds was measured during the austral summer of 2000 (December) using a method modified from Lobban (1978). Three *M. pyrifera* individuals were selected from a kelp bed with bottom depths of 3 to 5 m. A 4 h experimental incubation period was considered sufficient based on studies that have observed the transport of radioactive ^{14}C and ^{32}P from mature blades and along the entire frond of *M. integrifolia* within 4 h (Schmitz & Srivastava 1979a,b).

A blade was selected from each of the 3 *Macrocystis pyrifera* individuals that was situated between 30 and 40 blades (1.5 to 1.8 m) from the apical meristem (Fig. 1). Blades from this section of the frond are typically colonised by *Membranipora membranacea* and other epifaunal species (Hepburn & Hurd 2005), but during the experiment, epifaunal cover was low and covered less than 5% of blades selected. One 8 l clear, polyethelene bag was sealed around selected blades with flagging tape at the base and top of the pneumatocyst, using SCUBA. In preliminary experiments, universal dye was added to the sealed bag

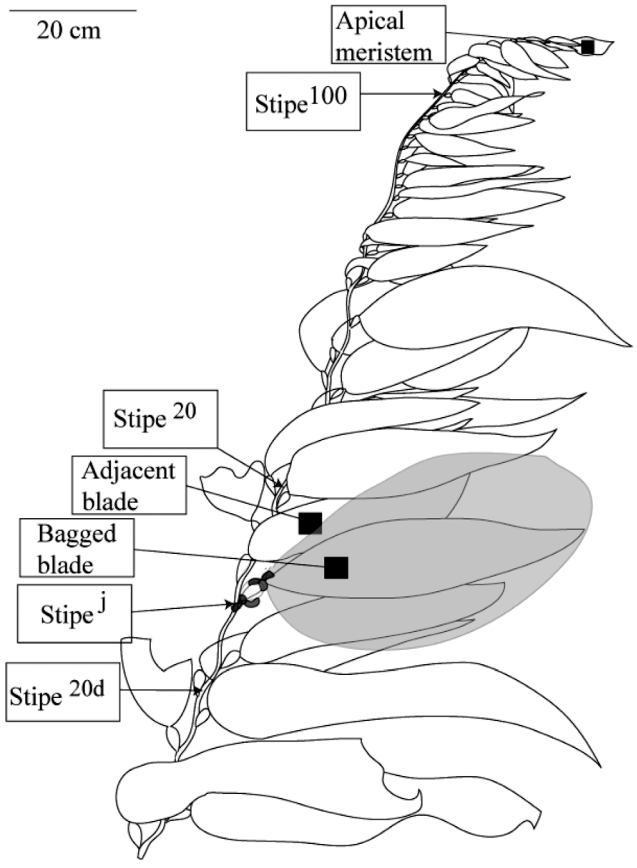

Fig. 1. *Macrocystis pyrifera.* Sampling plan of kelp fronds used for the *in situ* nitrogen transport experiment. Superscript numbers of stipe sample denote the distance (cm) from the bagged (grey shading) blade/stipe junction ($Stipe^{j}$) to the sample. $Stipe^{20d}$ indicates a stipe sample from 20 cm down the frond towards the holdfast.

and revealed no leakage of fluid contained within the bags. Each bag contained a screw-top bottle containing 20 ml of 6.25 mM $(NH_4)_2SO_4$ solution enriched by 10.4 % with ^{15}N. Bags were filled with 4.50 to 5.25 l of seawater to approx. 75 % of capacity to allow flexibility and water movement within the bag. Once the bag was sealed, the bottle was unscrewed through the side of the plastic bag releasing the ammonium to provide an initial concentration within the bag of approx. 50 µM above ambient. The plastic bag was then carefully shaken to mix the ammonium.

After 4 h, entire fronds with the bagged blades were cut at their base along with an adjacent frond from the same *Macrocystis pyrifera* individual that served as a control. Bags and the blades contained within them were then cut at the base of the pneumatocyst and placed, still sealed, aboard the boat. All cutting of *M. pyrifera* fronds was carried out in the water to prevent contamination on the boat deck. Samples of blade and stipe tissue along the 3 treatment and control fronds were taken using the sampling plan described in Fig. 1, and samples were placed in separate labelled zip-lock plastic bags. Lengths of stipe measuring approx. 5 cm were taken for stipe samples, while around 2 g (wet weight) of tissue was collected for blade and apical meristem samples from treatment and control fronds. After all blade and stipe samples had been collected, the volume of water in the bags was measured and blade tissue samples taken. All samples were then placed in an insulated bin for transport to the laboratory 1.5 h away. Water samples were taken from seawater surrounding each of the 3 sealed bags at the beginning of the incubation period and from water inside each bag 4 h later. The percentage of ammonium taken up by bagged *M. pyrifera* blades was estimated using the following:

$$\%NH_4^+ \text{ taken up} = \left[1-\left(\frac{S_f}{S_a+50\ \mu M}\right)\right]\times 100$$

Where, $S_f = NH_4^+$ concentration (µM) within the bags following the incubation period; $S_a = NH_4^+$ concentration of seawater surrounding bagged blades (ranged from 2–3 µM); and 50 µM = concentration of ^{15}N-labelled NH_4^+ within bags.

Blade and stipe tissue was then returned to the laboratory where it was dried at 80°C and ground using a mortar and pestle along with liquid nitrogen to aid grinding/homogenisation. Care was also taken not to contaminate tissue from parts of the stipe more distant from the bagged blades with more ^{15}N-enriched tissue by grinding samples further from the bagged blades first. Water samples were filtered (GF/C 1µm) and ammonium concentrations were determined using a Quickchem® 8000 automated ion analyser (Lachat Instruments).

Subsamples of dried, ground *Macrocystis pyrifera* tissue (1–2 mg) were combusted in a Carlo Erba NA1500 Elemental Analyzer (Carlo-Erba) interfaced to a Europa Scientific 20-20 update (SIRA10) continuous flow mass spectrometer (Sercon Australia). Corrections for drift were made automatically from a standard (EDTA, calibrated against NBS standards N1 and N2) with a known isotopic ratio every 5 samples. Ratios of ^{15}N:^{14}N are expressed in standard $\delta^{15}N$ (‰) notation with respect to air.

Differences in $\delta^{15}N$ between experimental groups were evaluated using paired *t*-tests ($\alpha = 0.05$). Tests for normality and equal variance were carried out to see if the criteria to perform parametric tests were fulfilled. All statistical analyses were carried out using the software package SigmaStat 2.03 (SPSS).

RESULTS

Laboratory feeding experiments

Treatment *Membranipora membranacea* tissue samples became enriched with ^{15}N from the phytoplankton food and displayed $\delta^{15}N$ values 26 times higher than control bryozoan tissue (Fig 2). An increase in the $\delta^{15}N$ value of 3‰ was observed for colonised *Macrocystis pyrifera* blade tissue from the treatment group compared to treatment bryozoan-free blade tissue but this difference was not significant (paired *t*-test, $t = -0.889$, $p = 0.424$). There was also no significant difference observed between control colonised and bryozoan-free blade tissue groups (paired *t*- test, $t = 0.0869$, $p = 0.935$).

In situ nitrogen transport in *Macrocystis pyrifera* fronds

Macrocystis pyrifera blade tissue from within the bags into which ^{15}N-labelled ammonium was released became highly enriched with the ^{15}N tracer, having $\delta^{15}N$ values approx. 220 times higher than control blades from an adjacent frond (Fig. 3). The level of enrichment was, however, quite variable, ranging 250‰ between bagged blade values from each replicate frond. The bagged blades absorbed on average 89 ± 6% (SE; n = 3) of the ammonium available from seawater within the polythene bags. There was evidence that bagged blades exported ammonium to the stipe during the 4 h incubation period. $\delta^{15}N$ values of the treatment stipe tissue at the junction of the bagged blade and the stipe ($Stipe^{J}$) were significantly higher than control blade tissue (paired *t*-test, $t = 4.42$, $p = 0.005$). $\delta^{15}N$ values were almost 3 times higher in treatment stipe tissue located 20 cm down the frond towards the holdfast ($Stipe^{20d}$) but this difference was not statistically significant (paired *t*-test, $t = -2.24$, $p = 0.154$). There was, however, no evidence of any transport of the ^{15}N tracer along the stipe towards the frond apex ($Stipe^{100}$).

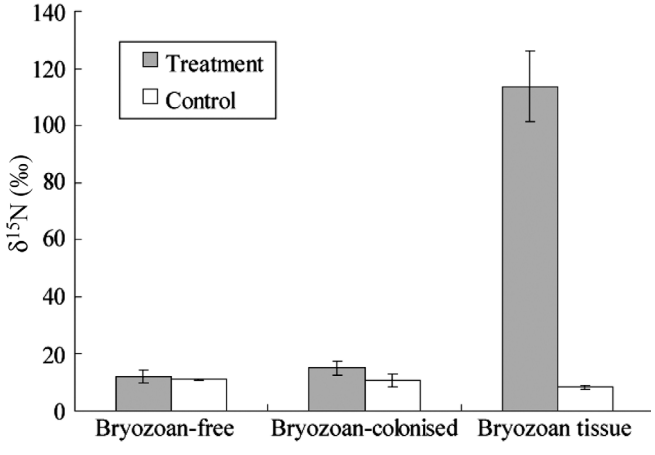

Fig. 2. *Macrocystis pyrifera* and *Membranipora membranacea*. $\delta^{15}N$ values of kelp blade tissue and that of bryozoan colonies following feeding experiments (8 h duration). Treatment samples had 1 ml of concentrated ^{15}N-enriched *Dunaliella tertiolecta* added at the beginning of the incubation period while nothing was added for control samples. Values indicate means ± 1 SE (n = 5)

DISCUSSION

The present study finds no strong evidence for the flow of nitrogen from *Membranipora membranacea* into underlying *Macrocystis pyrifera* tissue. The low degree of transmission of ^{15}N observed was probably due to the barrier formed by *M. membranacea* tissue to nitrogen uptake by underlying blade tissue (Hurd et al. 1994) and the low affinity for uptake of ammo-

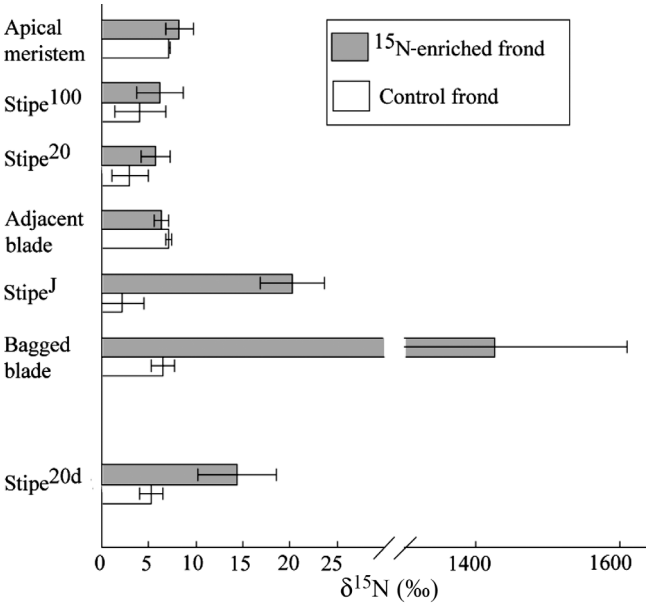

Fig. 3. *Macrocystis pyrifera*. $\delta^{15}N$ values of stipe and blade tissue from kelp fronds used to trace uptake and transport of nitrogen derived from ammonium *in situ*. Ten percent ^{15}N-enriched ammonium was made available to treatment fronds via a sealed bag that surrounded the bagged blade. Control frond tissue was taken from the same positions on an adjacent frond from the same individual. See Fig. 1 for position of samples. Values represent means ± 1 SE (n = 3). Superscript numbers of stipe sample denote the distance (cm) from the bagged blade/stipe junction ($Stipe^{J}$) to the sample

nium during the winter (Hepburn et al. 2006). Ammonium production by *M. membranacea* either as a result of sloppy feeding or due to excretion of waste products occurred on the upper surface of the colony away from the blade surface. Therefore, much of the ammonium produced by bryozoan colonies could be lost to the surrounding water column or to surrounding kelp tissue free from colonisation. The low degree of transmission may also be due to the high nitrogen content of blade tissue (1.6 to 2.2%) during this experiment and a corresponding low affinity for ammonium uptake during winter (Hepburn et al. 2006). It is possible that ammonium excretion may actually be harmful to *M. pyrifera* during periods when nitrogen is found in high concentrations. Excess ammonium can be toxic and could be the cause of reduced photopigments (Hepburn et al. 2006) and structural integrity of blade tissue beneath bryozoan colonies in *M. pyrifera* (Dixon et al. 1981, Hepburn 2003). The absence of *M. membranacea* on *M. pyrifera* during the summer (Hepburn & Hurd 2005) prevented a repeat of the experiment in summer, when the nitrogen status of blade tissue is much lower and rates of ammonium uptake can be between 4 and 6 times higher (Hepburn et al. 2006). During periods of low seawater nitrogen, it is likely that provision of nitrogen from *M. membranacea* to underlying kelp tissue will occur, but the significance of this nitrogen in reducing the effects of nitrogen limitation is yet to be determined.

This study finds further evidence that sheet-forming bryozoans like *Membranipora membranacea*, which provide a barrier to nitrogen uptake and are not abundant during periods of low nitrogen, are unlikely to provide significant benefits to host macroalgae via nitrogen provision (Hepburn & Hurd 2005, Hepburn et al. 2006). The timing of colonisation of kelp surfaces by sessile epifauna is critical in deciding the outcome of the relationship between epifauna and the macroalgae they colonise (Hepburn & Hurd 2005). *M. membranacea* typically has higher cover on *Macrocystis pyrifera* blades during the low-light, high-nitrogen winter months in southern New Zealand (Hepburn & Hurd 2005). This is consistent with colonisation patterns observed for *M. membranacea* in Californian kelp beds (Bernstein & Jung 1979, Yoshioka 1982) and is primarily a result of planktonic factors affecting the ability of the long-lived larvae of *M. membranacea* to settle on kelp surfaces (Yoshioka 1982b). During winter, the surface waters are well mixed and *M. membranacea* larvae are concentrated in the upper 5 to 10 m of the water column, allowing significant colonisation of the surfaces of *Macrocystis pyrifera*. When the seasonal thermocline forms in the early summer, *M. membranacea* larvae stop actively swimming and sink below 10 metres to avoid higher surface temperatures (Yoshioka 1982). This results in an inability to colonise shallow water kelp beds during the summer and reduces the probability of *M. membranacea* contributing a significant amount of nitrogen to macroalgal growth during the summer period of nitrogen limitation. Sites that exhibit high levels of colonisation during the summer (Hurd et al. 1994) are likely to be atypical and may be related to entrainment of larvae through localised physical processes (Dixon et al. 1981).

During *in situ* uptake and transport experiments, mature *Macrocystis pyrifera* basal blades were able to take up the majority of the ammonium available within the bags over 4 h. The higher affinity for ammonium uptake during *in situ* experiments was most likely a result of low internal pools of nitrogen within *M. pyrifera* during the summer compared to laboratory experiments that were conducted in winter when internal nitrogen pools are full (Hepburn et al. 2006). Nitrogen transport along the stipe was much slower than that observed for carbon and phosphorus (25 to 45 cm h^{-1}, Schmitz & Srivastava 1979a,b) with rates of 5 cm h^{-1} observed in some individuals and no transport of labelled nitrogen along the stipe away from bagged blades in others. Limited movement of ^{15}N-labelled nitrogen may reflect enzymatic rate limitation of assimilation of ammonium to amino acids and proteins before transport (Parker 1965, Manley 1983), or the time taken to saturate local nitrogen sinks within the blade before export of nitrogen to more distant sinks in apical and basal meristems can occur. Once reaching the stipe there was evidence of transport down the stipe towards the holdfast with ^{15}N enrichment of 4.5 to 17 ‰ being observed in treatment fronds. As the $\delta^{15}N$ values of blade tissue from bagged blades were still very high after the experiment, a longer incubation period may have allowed more mobilization of the ^{15}N tracer and a stronger signal on more apical sections of the frond.

Despite low levels of bryozoan colonisation observed during times of nitrogen limitation in the present study, the process of transporting nitrogen from older blades heavily colonised by epifauna to meristematic regions could be an important process for many associations between macrolgae and epifauna. High levels of *Membranipora membranacea* colonisation on *Macrocystis pyrifera* during time of nitrogen limitation and evidence of nitrogen provision to blade tissue has been observed in other geographic regions

(Hurd et al. 1994), and for other species within southern New Zealand such as *Ecklonia radiata* and *Carpophyllum flexuosum* (C. D. Hepburn pers. obs.). In the current study, hydroids were common on *M. pyrifera* during periods of low nitrogen, and had the potential to provide significant amounts of nitrogen to their kelp substratum during limitation (Hepburn & Hurd 2005). Future work should focus on transport of nitrogen from more diffuse hydroid colonies and how much nitrogen is maintained within diffusion boundary layers that surround kelp blades (Hurd 2000).

Nitrogen provided by sessile epifauna growing on mature blades that exhibit little or no growth can be exported to the stipe. This may provide nitrogen via long-distance transport to actively growing basal and apical meristems of *Macrocystis pyrifera* during times of nitrogen limitation, though the relative importance of this nitrogen source is not clear. This process may also be important for other macroalgae that transport solutes relatively long distances through symplasmic transport (Raven 2003) and may result in increased productivity of ecologically important kelp species when nitrogen is limiting to growth.

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