The *in situ* nitrogen (ammonium and nitrate) uptake kinetics of *Macrocystis pyrifera* (L.) C. Agardh: applications for integrated multi-trophic aquaculture (IMTA) in Big Glory Bay, Stewart Island, New Zealand

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Abstract

The *in situ* nitrogen (ammonium and nitrate) uptake kinetics of *Macrocystis pyrifera* and the potential for *M. pyrifera* to be used in an integrated multi-trophic aquaculture (IMTA) approach with salmon (*Oncorhynchus tshawytscha*) and mussels (*Perna canaliculus*) was investigated in Paterson Inlet, Stewart Island, New Zealand.

The *in situ* nitrogen uptake kinetics of *M. pyrifera* were determined using transparent polyethylene bags that were wrapped around *in situ M. pyrifera* blades or blade pieces and spiked with either ammonium or nitrate of varying concentrations. Experiments were conducted with both intact blades and blade pieces to assess the effect of tissue excision on macroalgal nutrient uptake. After being exposed to the nutrient solution, nutrient uptake rates were determined for each tissue type. *M. pyrifera* displayed rate-unsaturable uptake for ammonium at the studied concentrations (~80 µM) and uptake discontinuity for nitrate with uptake initially saturating (V_{max} = 31.67 µmol gdw^{-1}h^{-1}, K_s = 61.00 µM) and then displaying rate-unsaturable uptake thereafter. Tissue excision did not significantly affect *M. pyrifera* ammonium uptake and this was attributed to the long tissue recovery period (eighteen hours) allocated to the cut blades and the short incubation period which meant the loss of the capacity for translocation did not significantly affect uptake. The effect of tissue excision on *M. pyrifera* nitrate uptake could not be determined due to insufficient data.

To assess the suitability of *M. pyrifera* as an IMTA species, juvenile *M. pyrifera* were grown outside a salmon farm, a mussel farm and at a control site in both summer and autumn in Big Glory Bay or Glory Cove, Stewart Island, New Zealand. After the growing period (26-28 days), the *M. pyrifera* was harvested and growth rates, carbon and nitrogen status, nitrogen isotope (δ^{15}N) signatures and pigment concentrations were determined and compared. In summer there was some evidence of improved *M. pyrifera* growth at the salmon farm site and the *M. pyrifera* percentage nitrogen content and soluble ammonium concentration were higher at the salmon farm site compared to *M. pyrifera* that were grown at the mussel and control sites. Analysis of δ^{15}N signatures suggested that on average 68% of assimilated nitrogen was derived from the nitrogen in salmon pellets. However, in autumn, evidence indicated that salmon farm-derived nitrogen was a less important source of nitrogen as naturally occurring seawater nitrogen concentration was increasing. Analysis of δ^{15}N signatures suggested that on average only 26% of assimilated nitrogen was derived from the nitrogen in salmon pellets during autumn. Results demonstrate that *M. pyrifera* can take up nitrogen derived from the salmon farm but seasonal

differences in nitrogen demand by *M. pyrifera* suggest that nitrogen sequestration by this species will be greatest during the summer period.

The results from the *in situ* uptake experiments were combined with seawater chemistry and *M. pyrifera* nitrogen status data from the IMTA trials to answer two questions: (1) how long will it take for *M. pyrifera* to reach maximum tissue nitrogen content given the salmon farm nitrogen input rate and *M. pyrifera* ammonium uptake rate? and (2) how much *M. pyrifera* would need to be cultivated in summer and autumn to make a significant contribution to reducing nitrogen waste from the Kiwa 1 salmon farm, Big Glory Bay which produces 2,400 tonnes of salmon annually? By addressing these questions, insight was gained in terms of the short-term nitrogen enrichment buffering capacity of *M. pyrifera* as well as the amount of standing crop of *M. pyrifera* that would need to be cultivated in order to sequester the nitrogen inputs coming from the salmon farm. *M. pyrifera* takes approximately 63 hours to reach three percent nitrogen content (the maximum tissue nitrogen content for *M. pyrifera*) given the ammonium uptake rate of *M. pyrifera* and the rate of nitrogen input from the Kiwa 1 salmon farm. After accounting for nitrogen input from the Kiwa 1 salmon farm and the percentage nitrogen content of *M. pyrifera* from the salmon site, between 0.5 km^2 and 5.96 km^2 of *M. pyrifera* would be required to sequester all of the nitrogen inputs coming from the salmon farm depending on cultivation biomass and the season in which the *M. pyrifera* is grown. Such space requirements would be a significant area of Big Glory Bay and would require the closure of mussel and oyster farms and therefore total sequestration of Kiwa 1 salmon farm nitrogen inputs is not feasible. However, it was concluded that even if only a portion of the salmon farm-derived nitrogen could be sequestered by *M. pyrifera* cultivation, the IMTA approach could still be worthwhile. The co-cultured *M. pyrifera* could buffer against eutrophication and associated harmful algal blooms and provide oxygen, habitat, the potential for economic diversification and a point of difference in the market place.

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Chapter 1 Introduction

As human population size increases so does global demand for food and fish is an important protein-rich food source (Naylor et al. 2000; Rice and Garcia 2011). Many wild fish stocks are currently being harvested at unsustainable levels and will not be able to adequately meet the growing global demand for fish products (Naylor et al. 2000; Rice and Garcia 2011). The expansion of aquaculture has thus been proposed as a means to meet the growing global demand for fish whilst reducing harvest pressure on wild fish stocks (Naylor et al. 2000; Bostock et al. 2010; Merino et al. 2012). However, aquaculture is not a panacea for meeting increasing global fish demands as aquaculture can have associated adverse effects (Naylor et al. 2000; Bostock et al. 2010; Rice and Garcia 2011). These adverse effects can include disease and parasite transmission (Meyer 1991; Krkošek et al. 2007; Johansen et al. 2011), habitat loss (Naylor et al. 1998; Valiela et al. 2001), the spread of invasive species (Naylor et al. 2005; De Silva et al. 2009), genetic dilution and reduced fitness of wild fish populations (McGinnity et al. 2003; Naylor et al. 2005) and aquaculture-induced nutrient enrichment (Silvert 1992; Anderson et al. 2002; Mente et al. 2006).

Nutrient enrichment is commonly associated with the aquaculture of higher trophic level organisms such as fish, and finfish aquaculture-induced nutrient enrichment is a consequence of nutrient rich fish feed and fish excretory matter (Silvert 1992; Chen et al. 2003; Islam 2005; Mente et al. 2006; Fernandes et al. 2007; Wang et al. 2014a) (Figure 1.1). Fish feed and excretory matter are often high in inorganic nitrogen (Fivelstad et al. 1990; Kelly et al. 1994; Chen et al. 2003; Fernandes et al. 2007) and this can result in elevated dissolved inorganic nitrogen levels occurring in the waters surrounding marine open water finfish farms (Silvert 1992; Belias et al. 2003; Pitta et al. 2006; Navarro et al. 2008; Sanderson et al. 2008). Nitrogen-rich particulate matter is also a consequence of the fish feed and excretory matter and can settle and accumulate in the benthic environment (Silvert 1992; Mazzola et al. 2000; Holmer et al. 2005) (Figure 1.1).

Increased levels of anthropogenic inorganic nitrogen in the marine environment can have deleterious consequences. Because nitrogen is typically the key nutrient limiting primary production (Vitousek and Howarth 1991; Howarth and Marino 2006; Elser et al. 2007), anthropogenically elevated dissolved nitrogen levels can promote the occurrence of harmful algal blooms (HABs) (Hallegraeff 1993; Van Dolah 2000). These algal blooms can produce

toxins which in sufficient concentrations can kill fish, invertebrates, seabirds and marine mammals (Shumway 1990; Van Dolah 2000; Scholin et al. 2000; Shumway et al. 2003). Algal blooms can also result in reduced oxygen levels due to high algal respiration rates or the respiration of bacteria during algal decay (Hallegraeff 1993). Reduced oxygen levels can exert further stress on marine animals (Hallegraeff 1993). Additionally, the benthos can become inundated with nutrient rich organic particulate matter resulting in accumulation in the benthic environment. This accumulation of particulate matter can contribute to a reduced redox potential discontinuity (RPD) layer and the formation of bacterial mats (Silvert 1992; Wu et al. 1994; Mazzola et al. 2000). Bacterial activity can cause anoxia and the anaerobic generation of hydrogen sulfide and methane which is toxic to some marine organisms (Silvert 1992; Wu et al. 1994; Belias et al. 2003; Islam 2005).

The ecological and economic consequences associated with elevated dissolved nitrogen levels and the deposition of organic particulate matter can be severe. HABs can cause significant financial loss for marine farmers by causing shellfish toxicity or fish kills and when toxic shellfish are consumed, human illness or death can ensue (Chang et al. 1990; Shumway 1990; MacKenzie 1991; Hallegraeff 1993). Additionally, the accumulation of aquaculture particulate waste in the benthic environment can cause changes in benthic assemblages (abundance, biomass and species richness) typically away from less tolerant species towards more resilient species (Mazzola et al. 2000; Mirto et al. 2002; Yokoyama 2002; Kalantzi and Karakassis 2006; Yucel-Gier et al. 2007). Given the severity of these consequences and the intensification of aquaculture globally, solutions need to be devised and implemented to reduce or use aquaculture-derived nitrogenous waste if these problems and associated consequences are to be remediated. Potential solutions will be discussed in section 1.2 with a focus on integrated multi-trophic aquaculture (IMTA) as a nutrient enrichment remediation technique whereby macroalgae are used to take up aquaculture-derived nitrogen.

(1.1) Nitrogen in the marine environment and availability to macroalgae

(1.1.1) Ammonium (NH_4^+) and nitrate (NO_3^-) in the marine environment

Nitrogen is an essential element for macroalgae and the extent to which it is present can affect macroalgal growth, competition, succession, biomass and community structure (Lapointe and Tenore 1981; Fong et al. 1996; Valiela et al. 1997; Bracken and Nielsen 2004; Kim et al. 2007;

Aquilino et al. 2009; Ale et al. 2011). In the marine environment, two important inorganic nitrogen sources are available for use by macroalgae (and other phototrophs), ammonium (NH_4^+) and nitrate (NO_3^-) (Gruber 2008; Hurd et al. 2014). Nitrate is the most abundant fixed (by photoautotrophic organisms) form of nitrogen in the marine environment comprising around 88% of fixed oceanic nitrogen while ammonium makes up <0.3% of fixed oceanic nitrogen (Gruber 2008).

The supply of nitrogen in the coastal marine environment can vary in response to a myriad of biotic and abiotic factors. Nitrate levels can be influenced by the thermal stratification of the water column (Pennington and Chavez 2000; Whitney and Welch 2002), uptake by phytoplankton (Gruber 2008), atmospheric input (Krishnamurthy et al. 2010; Okin et al. 2011) and N_2 fixation via microbes (Galloway et al. 2003). Upwelling, vertical mixing, physical advection and tidal movements can supply nitrate from deeper waters to surface waters for use by phototrophs (Holloway et al. 1985; Keen et al. 1997). Biological activity is a major source of ammoniacal nitrogen in the euphotic zone of the coastal marine environment. Ammonium is regenerated via the bacterial decomposition of organic matter within sediments (Hurd et al. 2014) and macroalgal epifauna (Taylor and Rees 1998; Hepburn and Hurd 2006), bivalves (Kaspar 1985; Aquilino et al. 2009), heterotrophic plankton (Bode et al. 2004), fish (Bray et al. 1986; Wilkie 2002) and sea birds (Bosman et al. 1986) can all provide ammonium for use by macroalgae via their excretion.

The supply of both nitrate and ammonium in the marine environment can fluctuate due to nitrogen use by phototrophs and thermal stratification of the water column (Gruber 2008) resulting in significant seasonal variation in nitrate availability (Wheeler and North 1981; Wheeler and Srivastava 1984; van Tussenbroek 1989; Brown et al. 1997; Harrison and Hurd 2001; Hepburn and Hurd 2005; Stephens and Hepburn 2014). For temperate regions such as New Zealand, the nitrogen concentration in seawater is typically at minimal levels during late spring and summer and at maximal levels during late autumn and winter (Brown et al. 1997; Hepburn and Hurd 2005; Hepburn et al. 2007; Stephens and Hepburn 2014).

(1.1.2) Anthropogenic nitrogen in the marine environment

Human activities can be a major contributor of nitrogen to the marine environment and in some instances this can result in eutrophication. Eutrophication can be defined as:

The enrichment of water by nutrients, especially nitrogen and/or phosphorus and organic matter, causing an increased growth of algae and higher forms of plant life to produce an unacceptable deviation in structure, function and stability of organisms present in the water and to the quality of water concerned, compared to reference conditions (Andersen et al. 2006).

Sources of eutrophication can include agricultural runoff, sewage outfalls and aquaculture operations (Silvert 1992; Bonsdorff et al. 1997; Anderson et al. 2002; Galloway et al. 2003; Billen et al. 2013) and the consequences of marine eutrophication can be deleterious. Eutrophication can alter ecosystem structure and function (Wu et al. 1994; Meyer-Reil and Köster 2000; Deegan et al. 2002) and negatively affect human health and commercial activities (Shumway 1990; Hallergraeff 1993; Galloway et al. 2003).

Around finfish farms (with often high numbers of fish in a confined area), elevated localised nitrogen levels can exist because of ammonium production as a result biological activity (Silvert 1992; Handy and Poxton 1993; Wu et al. 1994; Belias et al. 2003; Pitta et al. 2006; Mantzavrakos et al. 2007; Sarà 2007; Sanderson et al. 2008). The elevated nitrogen levels are due to nitrogen leaching from the fish feed and also the excretion of undigested nitrogen in the fish faeces or digested nitrogen across the fish gills, skin or via the urine (Handy and Poxton 1993; Chen et al. 2003; Fernande et al. 2007). The amount of nitrogen released into the surrounding environment can vary depending on the food type, feed wastage and feeding methods used (Handy and Poxton 1993; Chen et al. 2003; Fernande et al. 2007). It has been estimated that that between 52-95% of nitrogen from aquaculture feed inputs can be lost into the environment through the leaching of nitrogen from pellets or fish excretion (Handy and Poxton 1993). Such enrichment can cause or contribute to eutrophication which has the potential to negatively affect the surrounding ecosystem, human health and cause cultured fish and shellfish mortality which can ultimately affect the commercial viability of an aquaculture operation (Shumway 1990; Hallegraff et al. 1993; Folke et al. 1994; Bonsdorff et al. 1997; Buschmann et al. 2006).

(1.2) Solutions to aquaculture-induced nutrient enrichment

Given that marine eutrophication can negatively affect aquaculture activities and aquaculture can itself be a source of eutrophication, systems and techniques are employed to reduce

aquaculture-induced nutrient enrichment. In some instances, aquaculture operations use underwater cameras to monitor when the farmed fish have been sufficiently fed which prevents overfeeding and hence unnecessary nitrogen entering the environment (Ang and Petrell 1997). This underwater camera approach can be coupled with knowledge of different fish feeding methods and behaviors so pellets can be optimally applied reducing the amount of pellets that the fish miss while feeding (Ang and Petrell 1998). Diet manipulation, specifically producing highly digestible food with appropriate protein and energy balance, can result in reduced levels of particulate waste and dissolved nitrogen and phosphorous (Talbot and Hole 1994; Cho and Bureau 2002; Amirkolaie 2011). Appropriate farm site selection can be used to make use of local currents to distribute and consequently dilute waste (Ackefors and Enell 1994; Talbot and Hole 1994) and the ability to physically move a farm can help limit localised nutrient loading. It has also been suggested that the aquaculture sector needs to focus on 'farming down the food web' that is, farming lower trophic-leveled organisms as opposed to higher trophic-leveled organisms (Stergiou et al. 2009; Tacon et al. 2010). Culturing organisms of lower trophic levels not only requires less food inputs but there is also less production of nutrient rich waste (Tacon et al. 2010).

The approaches mentioned above seek to reduce the source of aquaculture-induced nutrient enrichment. However, there is also a method for reducing nutrient enrichment that utilises the nutrients originating from aquaculture. This method is integrated multi-trophic aquaculture (IMTA).

(1.2.1) Integrated multi-trophic aquaculture (IMTA) as a solution for aquaculture-induced nutrient enrichment

Integrated multi-trophic aquaculture is a method that has been used to assist in the remediation of aquaculture-induced nutrient enrichment (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2009). IMTA is an aquaculture technique involving the co-culture of organisms of differing trophic levels (Chopin et al. 2001; Troell et al. 2009). The purpose of this is to utilise nutrient rich waste products originating from the farming process, thereby reducing the level of nutrient enrichment and the occurrence of problems commonly associated with nutrient enrichment (Chopin et al. 2001; Troell et al. 2009). Typical IMTA arrangements (Figure 1.2) that have been researched include: fish-bivalve (Sarà et al. 2009; MacDonald et al. 2011), fish-

macroalgae (Troell et al. 1997; Zhou et al. 2006; Wang et al. 2014a), bivalve-macroalgae (Mao et al. 2009), fish-bivale-macroalgae (Neori et al. 2004; Troell et al. 2009), shrimp-bivalve (Jones et al. 2002) and shrimp-macroalgae (Marinho-Soriano et al. 2009; Khoi and Fotedar 2011). Research has also been conducted to assess the potential for using sea cucumbers in IMTA with mussels (Slater and Carton 2009; MacTavish et al. 2012) and fish (Hannah et al. 2013; Yokoyama 2013) and molluscs (abalone) with macroalgae (Robertson–Andersson et al. 2008; Nobre et al. 2010).

Macroalgae are commonly used in IMTA as they are able to take up, assimilate and store dissolved inorganic nutrients (NH_4^+ and NO_3^-) of aquaculture origin (Chopin et al. 2001; Harrison and Hurd 2001; Neori et al. 2004; Troell et al. 2009). Because macroalgae can take up and store aquaculture derived nitrogen, the risk of aquaculture-induced nutrient enrichment and associated consequences is reduced (Chopin et al. 2001; Troell et al. 2009). In addition, there is the potential for the production of an economically useful by-product as macroalgae are used in phycocolloid and fertilizer manufacture and macroalgal products are consumed as food and health supplements by both humans and animals (Radmer 1996; Zemke-White and Ohno 1999; Chopin et al. 2001; Neori et al. 2004; Smit 2004; Gutierrez et al. 2006; Flores-Aguilar et al. 2007; FAO, 2014).

Globally, IMTA has been applied in a variety of aquaculture operations (open water systems, enclosed floating systems and land based cultures) and has been researched within North America, Asia, the Middle East, South America and Europe (Chopin et al. 2001; Troell et al. 2009; Bostock et al. 2010). In China, Canada, the United Kingdom, Israel and South Africa, IMTA has been put into commercial practice (Troell et al. 2009; Bostock et al. 2010). Despite some success internationally, IMTA using macroalgae has received relatively little attention in New Zealand (National Institute of Water and Atmosphere [NIWA] 2007). Consequently, there is a need to research the potential for IMTA in New Zealand.

(1.3) Sequestration of nitrogen by macroalgae

Macroalgae have the ability to take up, assimilate and store natural and anthropogenic sources of inorganic nitrogen (NH_4^+ and NO_3^-) (Troell et al. 1999, Neori et al. 2000; Chopin et al. 2001; Harrison and Hurd 2001; Abreu et al. 2009; Mao et al. 2009; Hurd et al. 2014). Nitrogen sources are stored intracellularly within the cytoplasm and vacuole and as amino acids, proteins

and pigments (Bird et al. 1982; Rosenberg and Ramus 1982; Shivji 1985; Stengel and Dring 1998; Naldi and Wheeler 1999; Harrison and Hurd 2001; Liu and Dong 2001; Kim et al. 2007; Ribeiro et al. 2013; Hurd et al. 2014). Some macroalgae have the ability to perform 'luxury' uptake of nitrogen enabling them to take up more nitrogen than they require when nitrogen is plentiful, for use during times of nitrogen limitation (Topinka and Robbins 1976; Fujita 1985; Harrison and Hurd 2001; Paul and de Nys 2008; Angell et al. 2014). Ammonium does not appear to be toxic to most macroalgae as concentrations in excess of 100 μM can yield normal uptake rates (Lavery and McComb 1991; Hurd et al. 1994; Taylor et al. 1998; Runcie et al. 2003; Gevaert et al. 2007). However, high ammonium levels from sewage discharges have been found to be toxic for some brown macroalgae (*Hormosira banksii, Durvillaea potatorum*) affecting their cell division and germination which can cause changes in macroalgal assemblages whereby opportunistic macroalgal species can dominate (Adams et al. 2008).

Typically, macroalgae will display preferential uptake of ammonium as opposed to nitrate (Rosenberg and Ramus 1984; Pedersen and Borum 1997; Lotze and Schramm 2000; Harrison and Hurd 2001; Cohen and Fong 2004; Luo et al. 2012; Sánchez de Pedro et al. 2013). Preferential uptake of ammonium has been attributed to greater uptake efficiency as nitrate requires one extra step in its assimilation; NO_3^- must be reduced to NH_4^+ by nitrate reductase which requires greater energy expenditure (Rosenberg and Ramus 1984; Harrison and Hurd 2001). In addition, ammonium uptake is usually faster than nitrate uptake because surge uptake is possible for ammonium and can usually occur independent of light (Pedersen and Borum 1997; Dy and Yap 2001; Harrison and Hurd 2001). However, preferential uptake of ammonium does not always occur and in some instances uptake for both species of nitrogen can be comparable (Kraemer et al. 2004; Martínez and Rico 2004).

(1.4) Macroalgal aquaculture

Macroalgae have been utilised by humans for centuries but the aquaculture of macroalgae has occurred only in the last few hundred years (Nash 2011). It is understood that macroalgal aquaculture originated in Japan where macroalgae were farmed for human consumption (Nash 2011). More recently, macroalgae are farmed throughout the world (Food and Agriculture Organisation [FAO] 2014) with a great majority of macroalgal aquaculture coming out of Asia with Tanzania (Zanzibar) and the Solomon Islands also being important providers (FAO 2014).

Macroalgae are farmed for carrageenan and agar manufacture, their pharmaceutical and nutraceutical properties, as well as for food, fertiliser, aquaculture feed and cosmetics (FAO 2014). Globally, macroalgal aquaculture contributes around one quarter of total global aquaculture (Zemke-White and Ohno 1999; FAO 2014). The aquaculture production of macroalgae has more than doubled between the period 2000 and 2012 (FAO 2014). In 2014, 23.8 million tonnes of algae (mostly macroalgae) was farmed with a value of US$6.4 billion (FAO 2014).

When identifying and selecting a macroalgal species for IMTA, the physiological characteristics and market value of the seaweed need to be considered (Neori et al. 2004). Important physiological characteristics include: effective biofiltration capacity, fast growth rates, large nitrogen sinks (capacity for nitrogen storage), tolerance of environmental stressors, and ease of cultivation (Chopin et al. 2001; Harrison and Hurd 2001; Neori et al. 2004; Kang et al. 2013). Ensuring these physiological characteristics are met will enable the selected macroalgal species to efficiently take up and store aquaculture-derived nutrients. A high market value of the seaweed is important to enable the economic viability of the IMTA approach if the goal of the IMTA is not solely bioremediation (Neori et al. 2004; Troell et al. 2009).

(1.5) *Macrocystis pyrifera* (L.) C. Agardh – ecological importance and aquaculture potential

Macrocystis pyrifera (L.) C. Agardh (Figure 1.3) is a kelp species that has a bipolar distribution and is found on the western coast of North America, South America (Peru, Chile and Argentina), South Australia, South Africa, Southern New Zealand as well as islands of southern latitudes (Graham et al. 2007). Ecologically *M. pyrifera* is an important species and is considered an ecosystem engineer (*sensu* Jones et al. 1994; Jones et al. 1997). The presence of *M. pyrifera* in the coastal marine environment can affect understory algal communities and marine grazers (Arkema et al. 2009), provide substrate for epifauna (Hepburn et al. 2007; Cerda et al. 2010), shelter and foraging areas for marine organisms (Moreno and Jara 1984; DeMartini and Roberts 1990; Anderson 2001), support fisheries (Vásquez et al. 2014) and provide a climate buffer (Graham et al. 2007; Vásquez et al. 2014).

There is growing interest (particularly in Chile) in the farming and harvest of *M. pyrifera* (Gutierrez et al. 2006; Buschmann et al. 2008; Vásquez et al. 2014; Correa et al. in press).

Interest has arisen in part because of the physiological characteristics of *M. pyrifera* as well as the potential for economic value. Research has revealed that *M. pyrifera* is able to efficiently sequester aquaculture derived nitrogen (Buschmann et al. 2008). The ability of *M. pyrifera* to sequester large amounts of nitrogen is a result of its fast growth rate and the large size it can achieve. Depending on aquaculture conditions, *M. pyrifera* can reach 1.75-14m in length and have a biomass of 14-80 kg m^{-1} of cultivation rope (Gutierrez et al. 2006; Westermeier et al. 2006; Macchiavello et al. 2010; Correa et al. in press). Because of the large size *M. pyrifera* can reach, there is the potential for *M. pyrifera* to be a substantial nitrogen sink (Buschmann et al. 2008). In addition, *M. pyrifera* is simple to culture (Westermeier et al. 2006; Macchiavello et al. 2010) and is capable of luxury uptake of nitrogen enabling *M. pyrifera* to take up more nitrogen than it requires when nitrogen levels are elevated, for use during periods of nitrogen limitation (Gerard 1982b; Shivji 1985; Zimmerman and Kremer 1986). With respect to economic value, in the past, harvested *M. pyrifera* was used primarily for alginate manufacture and had relatively low value (Troell et al. 2009). However, recently *M. pyrifera* has been farmed to provide higher value products including abalone and urchin feed, edible food products and organic fertilizers (Gutierrez et al. 2006; Flores-Aguilar et al. 2007).

Given the ability of *M. pyrifera* to effectively sequester nutrients as well as its potential for ecological and economic value, there are opportunities for *M. pyrifera* aquaculture to be effectively utilised in New Zealand. *M. pyrifera* could be used effectively to assist in nutrient enrichment remediation in New Zealand's mussel and salmon aquaculture industries. There could also be potential economic opportunities with the emerging pāua (*Haliotis iris*) (abalone) farming industry. Furthermore, farming *M. pyrifera* could assist in protecting wild *M. pyrifera* populations in New Zealand. Recently, *M. pyrifera* has been added to the New Zealand quota management system (QMS) so interest concerning wild *M. pyrifera* harvest may increase. The prospect of wild *M. pyrifera* harvest has generated concern from some stakeholder groups because of the importance of *M. pyrifera* as an ecosystem engineer. Given the physiological characteristics of *M. pyrifera*, its economic potential and the need to preserve wild *M. pyrifera* populations, *M. pyrifera* has been selected as the study species for this research.

(1.6) Research objectives, rationale and hypothesis

Given that nutrient enrichment and associated eutrophication are potential consequences of finfish aquaculture and can negatively affect the marine environment, it is important to progress towards the remediation of anthropogenic nutrient enrichment in New Zealand's coastal marine environment. In New Zealand however, IMTA is still in its early research stage and there is thus much scope for research in this area. To understand the extent to which a macroalgal species can be used in IMTA, it is important to have an understanding of the macroalgae's ecophysiology including the nutrient uptake kinetics of the species of interest (Harrison and Hurd 2001; Neori et al. 2004; Kang et al. 2013). This thesis combines data on the *in situ* nitrogen uptake kinetics of *M. pyrifera* with data from IMTA trials with salmon to assess the short-term buffering capacity of *M. pyrifera* (with respect to salmon farm nutrient inputs) and secondly, determine the amount of *M. pyrifera* that would need to be farmed to sequester the nutrient inputs originating from a salmon farm in Big Glory Bay, Stewart Island, New Zealand.

Chapter two investigates the *in situ* nitrogen uptake kinetics of *M. pyrifera*. Typically, nutrient uptake experiments are performed *in vitro* using small pieces of macroalgal tissue (Peckol et al. 1994; Ahn et al. 1997; Phillips and Hurd 2004; Gevaert et al. 2007; Rees et al. 2007; Abreu et al. 2011; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedro et al. 2013). This approach lacks realism and accuracy can be questionable as the macroalgal tissue is damaged by excision (Wheeler 1979) and the potential for the translocation of nutrients is reduced (Penot and Penot 1979; Schmitz and Srivastava 1979; Manley 1981; Raven 2003, Hepburn et al. 2012). This chapter investigates the *in situ* nitrogen (ammonium and nitrate) uptake kinetics of *M. pyrifera* whilst simultaneously investigating the effect that tissue excision has on *M. pyrifera* nitrogen uptake. Transparent bags were wrapped around *in situ M. pyrifera* blades or blade pieces and ammonium or nitrate was added at a range of concentrations. Ammonium and nitrate uptake rates were determined for each tissue type and compared. Because excision can damage the macroalgal tissue and reduce the capacity for translocation, it was hypothesised that the excised pieces of *M. pyrifera* would have a reduced nitrogen uptake rate.

Chapter three documents the results of IMTA trials conducted with *M. pyrifera*, Chinook salmon (*Oncorhynchus tshawytscha*) and green-lipped (Greenshell™) mussels (*Perna canaliculus*). Juvenile *M. pyrifera* were grown on frames outside a salmon farm, a mussel farm and at a control site in both summer and autumn. After the growing period, the *M. pyrifera* were harvested and important IMTA parameters (growth, nitrogen status, ^{15}N:^{14}N and pigment

levels) were determined to assess the potential of *M. pyrifera* as an IMTA candidate. Because finfish and bivalve aquaculture can provide ammonium via animal excretion and leaching from fish food, it was predicted that *M. pyrifera* growth, nitrogen status and pigment levels would be elevated at the salmon and mussel sites relative to the control site especially during the summer low seawater nitrogen period.

Chapter four combines data from the *in situ* nitrogen uptake experiments and data gathered from the IMTA trials to address two questions: (1) how long will it take for *M. pyrifera* to reach maximum tissue nitrogen content given the salmon farm nitrogen input rate and *M. pyrifera* ammonium uptake rate? (2) how much *M. pyrifera* would need to be cultivated in summer and autumn to make a significant contribution to reducing nitrogen waste from the Kiwa 1 salmon farm, Big Glory Bay? Addressing these questions will provide insight into the short-term nitrogen enrichment buffering capacity of *M. pyrifera* as well as the amount of standing crop of *M. pyrifera* that would need to be cultivated in order to sequester the nitrogen inputs coming from the salmon farm. Answering these questions will give further insight into the feasibility of an IMTA approach with salmon and *M. pyrifera* in Big Glory Bay.

This research will help to identify whether *M. pyrifera* would be a suitable IMTA species for use in Big Glory Bay and will contribute to the growing body of IMTA research worldwide. Additionally, this research will be some of the first IMTA research (using macroalgae) to be conducted in New Zealand and will be the first IMTA research conducted in Big Glory Bay. The results from the *in situ* nitrogen uptake experiments will contribute further to the understanding of *M. pyrifera* physiology and will provide insights for consideration with respect to the experimental design of future nutrient uptake experiments.
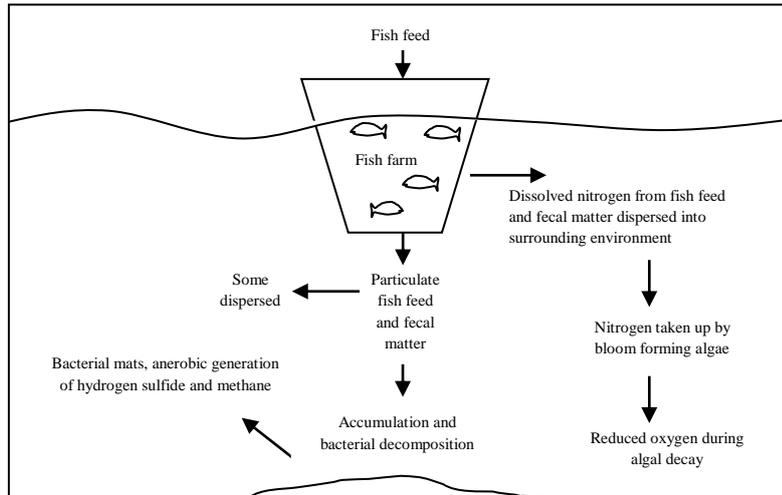
Figure 1.1: Conceptual diagram of a finfish farm outlining the origin of dissolved nutrients and particulate organic matter and the effect this can have on elements of the marine environment. Diagram produced from content in Silvert (1992), Mazzola et al. (2000) and Holmer et al. (2005).
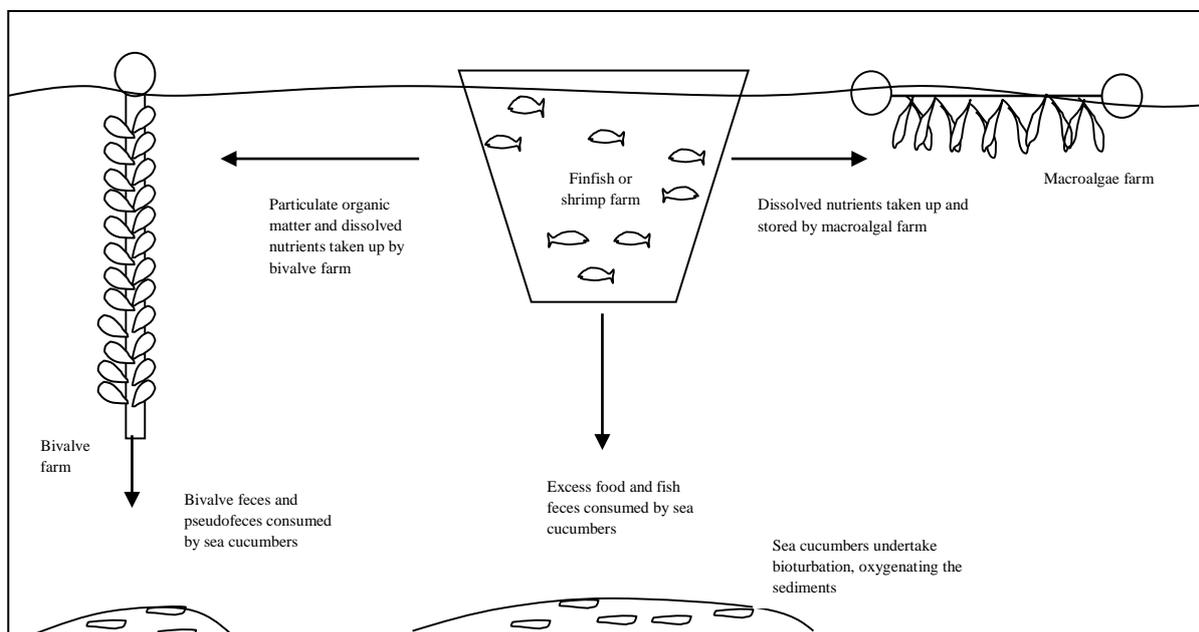
Figure 1.2: Conceptual diagram of integrated multi-trophic aquaculture involving finfish, bivalves, macroalgae and sea cucumbers. Diagram produced from content in Chopin et al. (2001) and Troell et al. (2009).

Figure 1.3: *Macrocystis pyrifera* from Paterson Inlet, Stewart Island, New Zealand. Photograph by Christopher Hepburn.

Chapter 2 *In situ* **ammonium (NH$_4^+$) and nitrate (NO$_3^-$) uptake kinetics of** *Macrocystis pyrifera*: **an analysis of alternative nutrient uptake methods**

(2.1) Introduction

(2.1.1) Nitrogen in the marine environment

For macroalgae, nitrogen is an essential element involved in the production of amino acids, purines, pyrimidines, amino sugars and amines (Hurd et al. 2014). In the marine environment, ammonium (NH$_4^+$) and nitrate (NO$_3^-$) are two important nitrogen species available for use by macroalgae (Gruber 2008; Hurd et al. 2014). In seawater, the concentration of these forms of nitrogen can vary in response to a number of biotic and abiotic factors (section 1.1) and changes in nitrogen availability can influence macroalgal growth, competition, succession, biomass and community structure (Lapointe and Tenore 1981; Fong et al. 1996; Valiela et al. 1997; Bracken and Nielsen 2004; Kim et al. 2007; Aquilino et al. 2009; Ale et al. 2011). Thus, understanding macroalgal uptake of nitrogen, specifically the nitrogen uptake kinetics of macroalgae, is of importance and can give insights into macroalgal competition for nutrient resources (Pedersen and Borum 1997; Campbell et al. 1999; Phillips and Hurd 2004), invasive macroalgal success (Campbell et al. 1999; Torres et al. 2004; Dean and Hurd 2007) and how macroalgae may respond to natural (seasonal), episodic and anthropogenic changes in nitrogen levels (Rosenberg et al. 1984; Lotze and Schramm 2000; Phillips and Hurd 2004; Torres et al. 2004; Gil et al. 2005; Wang et al. 2014b).

(2.1.2) Factors influencing macroalgal nutrient uptake kinetics

The rate at which macroalgae can take up inorganic nitrogen can be influenced by many physical and biological factors. Physical factors that can affect nutrient uptake rates include: light (irradiance) (Gerard 1982a; Nishihara et al. 2005), water movement (Kregting et al. 2008), temperature (Harlin and Craigie 1978; Nishihara et al. 2005), seasonality and zonation (Phillips and Hurd 2004), nitrogen type (ammonium, nitrate, nitrite, urea) and concentration (Wallentinus 1984; Dy and Yap 2001; Phillips and Hurd 2004) and frequency of nutrient exposure (single treatments or pulses) (Rosenberg et al. 1984). Biological factors that can affect nutrient uptake include nutritional past history (D'Elia and DeBoer 1978; Rees et al. 2007), macroalgal tissue type (Gerard 1982a), thallus morphology and the surface to volume ratio of

the macroalgal species (Wallentinus 1984; Pedersen et al. 2004). It is also possible that macroalgal epifauna can influence nutrient uptake positively through their provision of nitrogenous waste (Hepburn and Hurd 2005) and negatively through their physical presence which can provide a barrier for nutrient uptake (Hurd et al. 1994).

(2.1.3) Modelling nutrient uptake

Macroalgal nutrient uptake ability is commonly examined using the Michaelis-Menten model (D'Elia and DeBoer 1978; Gerard 1982a, Wallentinus 1984; Phillips and Hurd 2004; Gìl et al. 2005; Kregting et al. 2008; Wang et al. 2014b) which was originally produced to describe the rate of enzymatic reactions (Michaelis and Menten 1913; Johnson and Goody 2011). For use with macroalgae, the Michaelis-Menten model involves plotting macroalgal uptake rate against a range of substrate concentrations with the relationship between uptake rate and substrate concentration being a hyperbolic curve (Figure 2.1) (Gerard 1982b; Wallentinus 1984; Phillips and Hurd 2004; Kregting et al. 2008; Wang et al. 2014b). From this curve the half-saturation constant (K_s), maximal uptake rate (V_{max}) and the initial slope (α) of the curve are determined (Figure 2.1). The parameter K_s is indicative of the substrate concentration where uptake rate is half its maximum and denotes the affinity of the macroalgal tissue for a particular ion whereby low values denote a high affinity (Harrison and Hurd 2001; Hurd et al. 2014). K_s can be a useful proxy for predicting how an increase in nutrient availability may increase macroalgal nutrient uptake and growth (Phillips and Hurd 2004). V_{max} is the maximum uptake rate at a saturating substrate concentration (Harrison and Hurd 2001). A large V_{max} indicates an ability to rapidly take up nutrients when nutrient concentrations are high (Harrison and Hurd 2001). The initial slope (α) of the hyperbolic curve is useful for comparing the uptake ability of macroalgae at low substrate concentrations with a steep α indicating a high affinity for a specific nutrient at low concentrations (Harrison et al. 1989; Harrison and Hurd 2001).

In some instances, uptake demonstrates a rate-unsaturated (linear) response for a given nutrient (Probyn and McQuaid 1985; *Harrison* et al. *1986;* Taylor et al. 1998; Smit 2002; Phillips and Hurd 2004; Abreu et al. 2011; Wang et al. 2014b). In these cases it is not possible to determine V_{max} so the macroalgal uptake rate needs to be documented for a given substrate concentration or the slope of the regression can be used to find the uptake rate (Harrison and Hurd 2001).

(2.1.4) Pathways of nutrient uptake

Lobban and Harrison (1994) document the mechanisms for ion transport into macroalgal cells. For an ion or molecule to enter a macroalgal cell it must first move across the concentration boundary layer of water that surrounds the cell, then through the cell wall and plasmalemma before entering the cytoplasm. The mechanisms by which a substance moves across the plasmalemma can include passive transport, facilitated diffusion, active transport or a combination of these mechanisms. Passive transport involves uncharged molecules moving across the plasmalemma down a concentration gradient and charged molecules moving across the plasmalemma down an electrochemical-potential gradient. Passive transport does not require energy expenditure by the cell. Facilitated diffusion involves molecules and ions moving across the plasmalemma through a carrier protein intermediary. This too happens across an electrochemical gradient without the expenditure of energy. Active transport involves molecules or ions moving against an electrochemical-potential gradient. Molecules and ions move into the cell via ATPases with the expenditure of ATP energy.

The mechanism by which a molecule or ion enters a cell will affect the nutrient uptake kinetics of a macroalgal individual. It is understood that if a species of macroalgae exhibits rate-unsaturable uptake kinetics for ammonium (which is often the case (Probyn and McQuaid; 1985; Harrison et al. 1986; Taylor et al. 1998; Smit 2002; Phillips and Hurd 2004; Wang et al. 2014b)), the uptake of ammonium is via passive transport (Harrison and Hurd 2001). However, if ammonium displays rate-saturable uptake kinetics then ammonium uptake is presumed to be by active transport (Harrison and Hurd 2001). Uptake of nitrate by macroalgae generally exhibits rate-saturable uptake kinetics (Gerard 1982a; Wallentinus 1984; Thomas et al. 1985; Braga and Yoneshigue-Valentine 1996; Hurd et al. 1996; Torres et al. 2004; Phillips and Hurd 2004; Dean and Hurd 2007; Rees et al. 2007; Wang et al. 2014b) and it is likely that uptake is via active transport (Harrison and Hurd 2001). Following uptake, nitrogen is stored intercellularly in the vacuole or cytoplasm and as amino acids, proteins and pigments (Wheeler and North 1981; Bird et al. 1982; Rosenberg and Ramus 1982; Smit et al. 1998; Harrison and Hurd 2001; Hurd et al. 2014).

(2.1.5) Methods for estimating macroalgal nutrient uptake kinetics

There are two approaches used to estimate the nitrogen uptake kinetics of macroalgae: the stable-isotope method (Williams and Fisher 1985; Naldi and Wheeler 2002; Cohen and Fong 2004; Tarutani et al. 2004) and the method where the depletion of a nutrient from the surrounding seawater medium is measured colormetrically (Gerard 1982a; Rosenberg et al. 1984; Wallentinus 1984; Probyn and McQuaid 1985; Peckol et al. 1994; Abreu et al. 2001; Phillips and Hurd 2004; Gevaert et al. 2007; Rees et al. 2007; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedro et al. 2013). These approaches can be conducted *in vitro* (under laboratory conditions) or *in situ* (under field conditions).

The stable-isotope method involves subjecting macroalgal tissue to a nutrient solution enriched with the isotope of interest for a set period of time (Williams and Fisher 1985; Naldi and Wheeler 2002; Cohen and Fong 2004; Tarutani et al. 2005). Following the exposure time, tissue samples used in the experiment and reference tissue (not exposed to isotope enriched solution) are analysed using a mass spectrometer. The uptake rate of the isotope can then be determined by taking into consideration the change in the isotopic signature of the macroalgal tissue and the exposure time.

The approach which involves colormetrically measuring the depletion of a nutrient from a medium is most commonly used (Gerard 1982a; Rosenberg et al. 1984; Wallentinus 1984; Probyn and McQuaid 1985; Peckol et al. 1994; Phillips and Hurd 2004; Gevaert et al. 2007; Rees et al. 2007; Abreu et al. 2011; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedro et al. 2013) and there are two ways to utilise this approach: the time-course depletion approach and the multiple flask method. The time-course depletion approach involves placing macroalgal tissue into a solution spiked with the nutrient of interest and then taking water samples from the solution over a period of time (Probyn and Chapman 1982; Smit 2002; Abreu et al. 2011; Sánchez-Barredo et al. 2011). The depletion of the nutrient of interest produces a time-course for the uptake of the nutrient (Hurd et al. 2014). The multiple flask method involves setting up many containers with different concentrations of the nutrient of interest and then subjecting macroalgal tissue to the differing nutrient solutions for a set period of time (Peckol et al. 1994; Ahn et al. 1997; Phillips and Hurd 2004; Gevaert et al. 2007; Abreu et al. 2011; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedroet al. 2013). Following the exposure period, samples of the nutrient solution are compared with the starting concentration of the nutrient solution and the nutrient uptake rate is determined per gram of

macroalgal tissue. Data that displays saturation can have the Michaelis-Menten model applied and parameters V_{max}, K_s and α can be used to compare nutrient uptake.

These approaches can be performed both *in vitro* and *in situ* but typically the multiple flask method (discussed above) is used *in vitro*. Numerous small glass flasks or beakers are set up with a specific concentration of the nutrient of interest and either a small piece of macroalgal tissue or a whole macroalgal blade (Rosenberg and Ramus 1984; Peckol et al. 1994; Phillips and Hurd 2004; Gevaert et al. 2007; Abreu et al. 2011; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedro et al. 2013). After the exposure period, uptake rates and kinetic parameters are determined for the macroalgal tissue piece, blade or frond. An alternative *in vitro* approach is to use continuous flow culture systems (mesocosms) in which the macroalga of interest is allocated a container/chamber and supplied with a flow of seawater spiked with the nutrient of interest (Rosenberg et al. 1984). A modification of the Michaelis-Menten equation which includes flow data is then applied and kinetic parameters determined (Rosenberg et al. 1984). The laboratory conditions used in these *in vitro* approaches allow factors such as light, temperature and water movement to be kept constant.

The *in situ* nutrient uptake rates of macroalgae have also been examined using the 'multiple flask' method. However, instead of using flasks or beakers, individual blades or fronds of macroalgae are bagged or bottled up in the field and then subjected to a known concentration and volume of nutrient solution for a specified period of time (Gerard 1982a; Wallentinus 1984; Probyn and McQuaid 1985; Lavery and McComb 1991). Kinetic parameters are then used to analyse nutrient uptake (Gerard 1982a; Wallentinus 1984; Probyn and McQuaid 1985; Lavery and McComb 1991). The *in situ* approach does not allow the experimenter the same control over light, temperature and water movement that the *in vitro* approach provides. The experimental setup can also be time consuming and is a potential reason why the *in situ* approach is seldom used. However, the *in situ* approach does allow the macroalgae to be kept intact and provides realistic experimental conditions. The importance of these factors is discussed in section 2.1.6.

(2.1.6) *In vitro* vs. *in situ* **nutrient uptake experiments – potential problems with the** *in vitro* **approach**

Observation of the peer reviewed literature reveals that, typically, the nutrient uptake kinetics of macroalgae are estimated *in vitro* using small pieces of macroalgal tissue (see section 2.1.5) (Peckol et al. 1994; Ahn et al. 1997; Phillips and Hurd 2004; Gevaert et al. 2007; Rees et al. 2007; Abreu et al. 2011; Sánchez-Barredo et al. 2011; Luo et al. 2012; Sánchez de Pedro et al. 2013) which allows for convenient experimental setup and accurate control of experimental conditions namely light, temperature and water movement. However, it can be argued that *in vitro* experiments lack realism and this can raise questions as to whether results of *in vitro* uptake experiments can be extrapolated and applied to what is observed in nature.

Doubts concerning the applicability of *in vitro* uptake experiments can arise because the macroalgal tissue used in these experiments is often damaged by tissue excision. Consequently, the nutrient uptake parameters determined may not be a true representation of nutrient uptake (Wheeler 1979). Additionally, some macroalgae (particularly large and complex Laminariales) can possess mechanisms for long distance transport (translocation) of substances to distant sinks (Penot and Penot 1979; Schmitz and Srivastava 1979; Manley 1981; Raven 2003, Hepburn et al. 2012). Because small tissue pieces are commonly used in *in vitro* experiments, there is the potential for reduced translocation capacity. Conversely, *in situ* nutrient uptake experiments involving whole intact blades or fronds of macroalgae would theoretically allow for the unimpeded translocation of substances within the macroalgal individual and hence provide a greater degree of realism. Performing *in situ* uptake experiments could thus give added insight into the nutrient uptake ability of a macroalgal species.

Limited research has been undertaken to determine the *in situ* nutrient uptake kinetics of macroalgae. However, when these experiments have been performed, useful insights with respect to macroalgal nutrient uptake have been drawn. For example, Gerard (1982a) documented the *in situ* nitrate uptake kinetics of *M. pyrifera*. Gerard (1982a) placed transparent and black polyethylene bags around different types of *M. pyrifera* blades *in situ*. Gerard (1982a) was able to conclude that different types of *M. pyrifera* blade yielded different uptake rates and uptake rates varied with respect to the presence or absence of light. Probyn and McQuaid (1985) used a similar approach to determine the *in situ* ammonium and nitrate uptake rates of whole fronds of *Ecklonia maxima*. Probyn and McQuaid (1985) placed transparent polyethylene bags around whole *in situ* fronds of *E. maxima* and were able to conclude that *E.*

maxima nitrate uptake did not saturate at their studied concentrations (20μg-at N l^{-1}) and that *E. maxima* displayed a slight preference for ammonium.

Although the experimenter has less control over experimental parameters in *in situ* uptake experiments (it is harder to control light, water movement and temperature) and there is more margin for error, it can be argued that *in situ* experiments are more realistic and could be more applicable to what is observed in nature.

(2.1.7) Study species - *Macrocystis pyrifera*

Macrocystis pyrifera has the capacity for long distance transport of nutrients to distant sinks (Parker 1966; Schmitz and Srivastava 1979a; Manley 1981; Raven 2003, Hepburn et al. 2012) and thus excising blades for nutrient uptake experiments could affect macroalgal uptake rates for this species. Excising (damaging) *M. pyrifera* tissue has been found to negatively affect methylamine (an ammonium analogue) uptake (Wheeler 1979). The physiological characteristics, morphological complexity and capacity for long distance transport makes *M. pyrifera* an ideal species for determining the effect of macroalgal tissue excision on *in situ* nutrient uptake and consequently has been selected as the study species for this chapter.

(2.1.8) Experimental aims, approach and hypothesis

Given the potential for *in situ* uptake experiments to yield additional insight into the nutrient uptake kinetics of macroalgae and that excising macroalgae for *in vitro* experiments could affect the experimental outcome, this chapter aims to: (1) determine the *in situ* NH_4^+ and NO_3^- uptake kinetics of *M. pyrifera* and (2) determine the effect of tissue excision on the *in situ* NH_4^+ and NO_3^- uptake kinetics of *M. pyrifera*. To achieve this, the *in situ* bagging approach was used (Gerard 1982a; Probyn and McQuaid 1985). Two different forms of *M. pyrifera* tissue, an attached blade and an excised piece of blade, were added to individual bags spiked with differing concentrations of either NH_4^+ or NO_3^-. Following the exposure period, the uptake kinetics of the different forms of *M. pyrifera* tissue were determined and compared for each nutrient and tissue type. Because excising macroalgal tissue for uptake experiments can damage the individual and potentially prevent the translocation of nutrients, it was

hypothesised that the attached *M. pyrifera* blade and blade piece would differ in their nutrient uptake rates. Specifically, it was predicted that the attached blade would have a higher uptake rate than that of the excised blade.
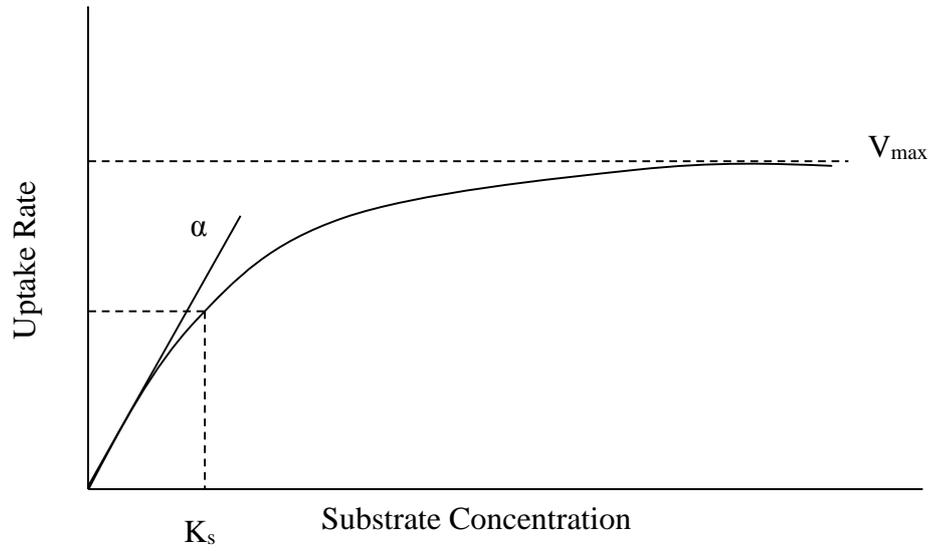
Figure 2.1: Hyperbolic curve outlining parameters V_{max}, K_s and α typically used in determining the nutrient uptake kinetics of macroalgae.

(2.2) Methods

(2.2.1) Preliminary time course experiments

Prior to undertaking the *in situ* uptake experiments, preliminary time course experiments were conducted between 1000 and 1400 h in summer 2013 at Portobello, Otago, New Zealand (45°49'40.40"S, 170°38'27.36"E). These preliminary experiments were performed to establish familiarity with the *in situ* method and enable an appropriate experimental period to be determined. To perform these time course experiments, six mature canopy blades were harvested from six *M. pyrifera* fronds. Each blade was individually allocated to clear 30 L polyethylene bag that was filled with eight litres of unfiltered seawater and placed into a 54 L fish bin for ease of handling. A 25 ml container with a 4 ml solution of KNO_3 or $2NH_4Cl$ 0.1 M was then added to the bag making the nitrogen concentration in the bags ~50 μM or ~100 μM respectively (n = 3 for both nitrogen treatments). The containers were opened and the bags were mixed by hand for 15-30 seconds. To monitor the depletion of the NH_4^+ or NO_3^- from the bags, a 10 ml water sample was taken from the bag at 0, 10, 20, 30, 40, 60, 80 and 100 minutes. Periodic shaking of the bag was performed to inhibit boundary layer formation (Wheeler 1980; Gerard 1982b; Hurd et al. 1996).

Following sample collection, the samples were filtered (Whatman™GF/C) and frozen for later determination of either NH_4^+ or NO_3^-. After the water samples were defrosted, the NH_4^+ or NO_3^- in each sample was determined using a Quickchem® 8500 automated ion analyser (Lachat Instruments, Milwaukee, USA). NH_4^+ or NO_3^- concentrations in each water sample were estimated using the methods outlined in Strickland and Parsons (1968) and Lachat Instruments (2008). Ammonium concentration in the seawater samples was estimated using the indophenol-blue method. Nitrate concentration in the seawater samples was estimated by reduction with a copper-coated cadmium column. Following sample analysis, the changing NH_4^+ or NO_3^- concentrations in the bag was graphed against time. This produced a time course of NH_4^+ or NO_3^- depletion in the bag due to uptake by the *M. pyrifera* blade.

(2.2.2) Experimental location, blade pre-treatment and experimental procedure

The methods developed by Gerard (1982a) formed the basis of the methods for studying the *in situ* NH_4^+ and NO_3^- uptake kinetics of *M. pyrifera*. *In situ* uptake experiments were conducted

between 1000 h and 1400 h in late spring and summer, at Golden Bay, Stewart Island, New Zealand (46°54'13.16"S, 168°7'20.85"E) (Figure 2.2). There were two treatments, blades that had been removed and cut and blades that were left intact on the *M. pyrifera* individual.

The day before undertaking the uptake experiments, fifteen *M. pyrifera* blades were harvested from fifteen different individuals. These fifteen blades were to be used the following day in the uptake experiments where excised tissue was required. The harvested blades were selected from the canopy region of the *M. pyrifera* individual, approximately ten–fifteen blades away (depending on blade condition) from the apical meristem of the individual. These *M. pyrifera* blades were cut with a razor blade into a rectangle shape with an average area of 88 cm^2, SE ± 3.2 cm^2. The cut pieces of *M. pyrifera* were held in a fish bin with seawater overnight allowing for recovery from emersion and wounding. Recovery time was at least 18 hours. Allowing for recovery from emersion and wounding is best practice for *in vitro* experiments where tissue pieces are used (Wheeler 1979; Phillips and Hurd 2004).

Experiments on both the cut and intact blades were run over three days in spring and summer. Before any nutrient uptake experiments were performed, a random number generator was used to assign each treatment (nitrogen source, concentration and cut/intact) to an experimental day and time (either late morning or early afternoon). This ensured that bias was reduced because certain experimental days or times could produce preferable conditions (light, temperature, water movement) for nitrogen uptake.

Twelve polyethylene bags were set up for each experimental run and there were two runs per day. Bags allocated a cut piece of *M. pyrifera* tissue were given four litres of seawater and a 0-20 ml aliquot of either $2NH_4Cl$ or KNO_3 0.01 M, sealed in 25 ml container. Bags that were to be assigned an attached *M. pyrifera* blade were given eight litres of seawater and a 0-40 ml aliquot of either $2NH_4Cl$ or KNO_3 0.01 M sealed in one or two 25 ml containers. These bag arrangements allowed the ammonium and nitrate concentrations in the bags to be ambient (1-2 μM) through to ~80 μM. Different volumes of seawater (four or eight litres) were added to the differing treatments to provide similar tissue weight to volume ratios for each treatment. Each of the polyethylene bags had a small cut in its corner that was sealed with a plastic bag clip. This cut allowed water samples to be taken from the bag. For a diagram of experimental setup see Figure 2.3.

After the bags had been assigned their various nitrogen treatments, they were transported from the shore to the experimental site by kayak or wading. Bags that were assigned a cut piece of *M. pyrifera* were sealed with a cable tie or insulation tape and a piece of cord (20 cm) was then used to fasten the bag to an *M. pyrifera* stipe at the experimental site. For those bags assigned an attached *M. pyrifera* blade in summer experiments, cable ties were used to seal the bag around the attached *M. pyrifera* pneumatocyst. However, there was concern that the cable tie could damage the pneumatocyst and consequently, in the spring experiments insulation tape was used to seal the bag around the *M. pyrifera* pneumatocyst. The attached blades were ten–fifteen blades down from the apical meristem (depending on blade condition and epiphyte presence). Those blades that were too heavily colonised with epifauna were excluded, as there was the potential for them to affect nitrogen uptake (Hurd et al. 1994; Hepburn and Hurd 2005). During the experiments, six control bags with no *M. pyrifera* tissue present were set up to determine the change in seawater NH_4^+ and NO_3^-, independent of the influence of *M. pyrifera*. Control bags had four litres of seawater added to them with appropriate volumes of $2NH_4Cl$ or KNO_3 0.01 M to make the desired concentrations.

When all of the bags were set up (twelve each experimental run, two runs each day), the containers holding the various nutrient types and volumes were opened through the bag. After opening each container, the bags were agitated (mixed) for fifteen or thirty seconds in summer and spring respectively. Different mixing periods occurred each season because after the summer experiments were completed there was concern that fifteen seconds was an insufficient period of time to allow for adequate mixing of the bag. A 10 ml aliquot was then taken from the cut in the corner of the bags via a syringe. This water sample was for the determination of the initial nitrogen concentration in the bag. After an experimental period of 60 minutes, a final 10 ml water sample was taken. When the experiments were completed for the day, water samples were frozen for later determination of nitrogen content. The *M. pyrifera* blades or tissue pieces were placed in labelled bags and weighed to determine wet weight. The collected *M. pyrifera* tissue was also dried at 50ºC for seven days to determine tissue dry weight. Water samples were filtered (Whatman™GF/C) before they were analysed for NH_4^+ or NO_3^- using an automated ion analyser using methods outlined in section 2.2.1.

During each experimental period a water temperature reading and surface light measurement was taken. Light measurements were taken using a LI-250A light metre (LI-COR, Nebraska

USA) in summer and a HOBO light logger (Onset, Massachusetts USA) placed on a nearby rock in spring.

(2.2.3) Calculation of *in situ* nitrogen uptake rates and kinetic parameters

NH_4^+ and NO_3^- uptake rates were calculated using the equation $V = (S_i - S_f) \times vol/ t \times dw$ (Hurd et al. 2014). V is the uptake rate ($\mu mol \cdot dry\ wt^{-1} \cdot h^{-1}$), S_i and S_f are the initial and final substrate concentrations (μM) respectively, vol is the volume of the substrate (L), t is the time (hours) and dw is the dry weight (g) of the *M. pyrifera* tissue.

M. pyrifera exhibited rate-unsaturable uptake kinetics for NH_4^+ and was analysed using a linear regression (Zar 1996). For NO_3^-, *M. pyrifera* exhibited rate-saturable uptake kinetics and consequently the Michaelis-Menten model $V = V_{max} \times (S/(K_s + S))$ was applied. V is the uptake rate ($\mu mol \cdot dry\ wt^{-1} \cdot h^{-1}$), S is the substrate concentration (μM), V_{max} is the maximum uptake rate at saturating concentration ($\mu mol \cdot dry\ wt^{-1} \cdot h^{-1}$) and K_s is the half-saturation constant (μM) (Hurd et al. 2014). The initial slope (α) of the uptake rate vs substrate concentration curve was calculated as V_{max}/K_s (Kopczak 1994). Statistical analysis and fitting of the Michaelis-Menten model was performed with Graphpad Prism $6^{®}$.
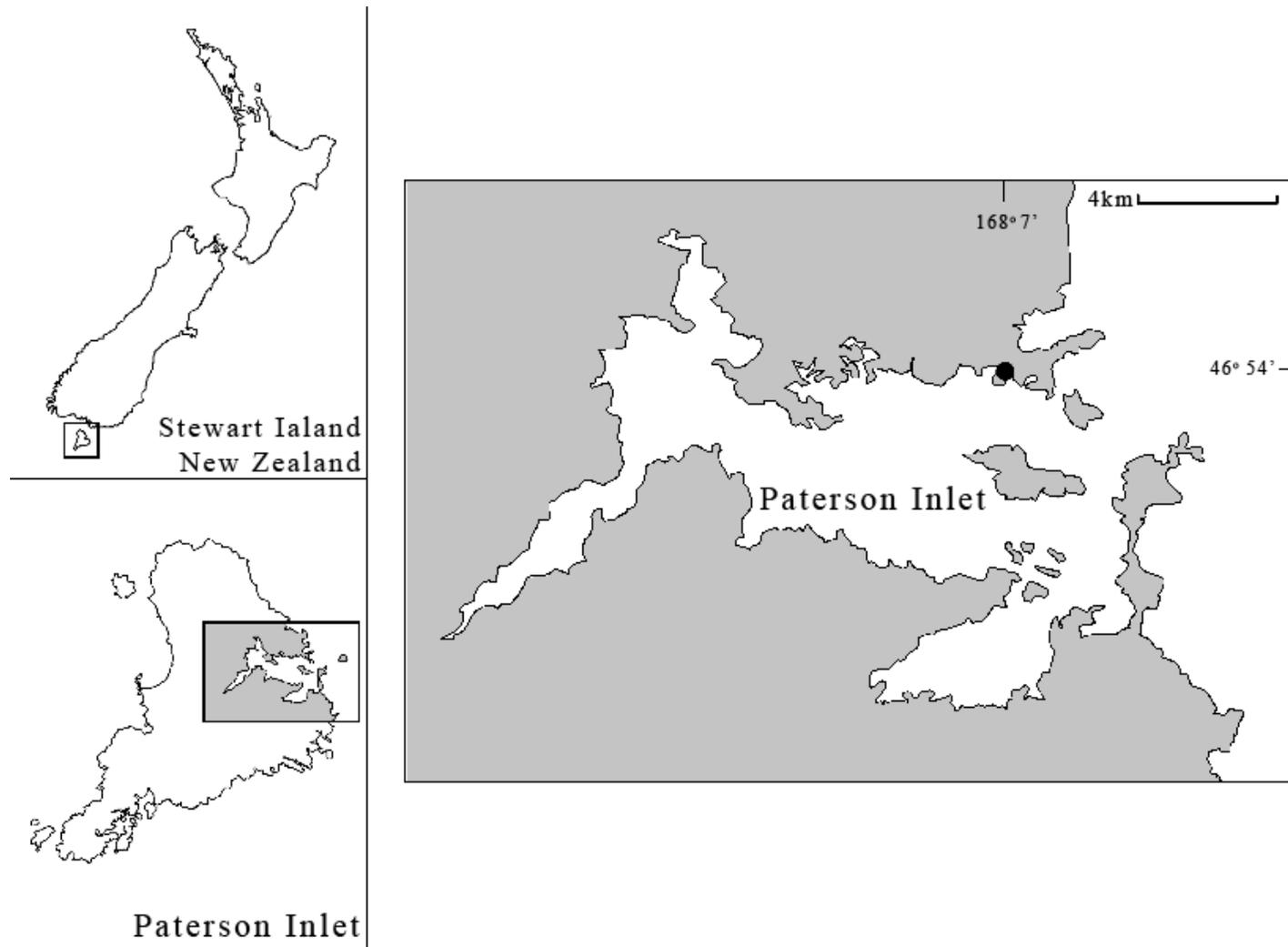
Figure 2.2: Map of New Zealand, Stewart Island and Peterson Inlet (Stewart Island) showing the Golden Bay study site (●).
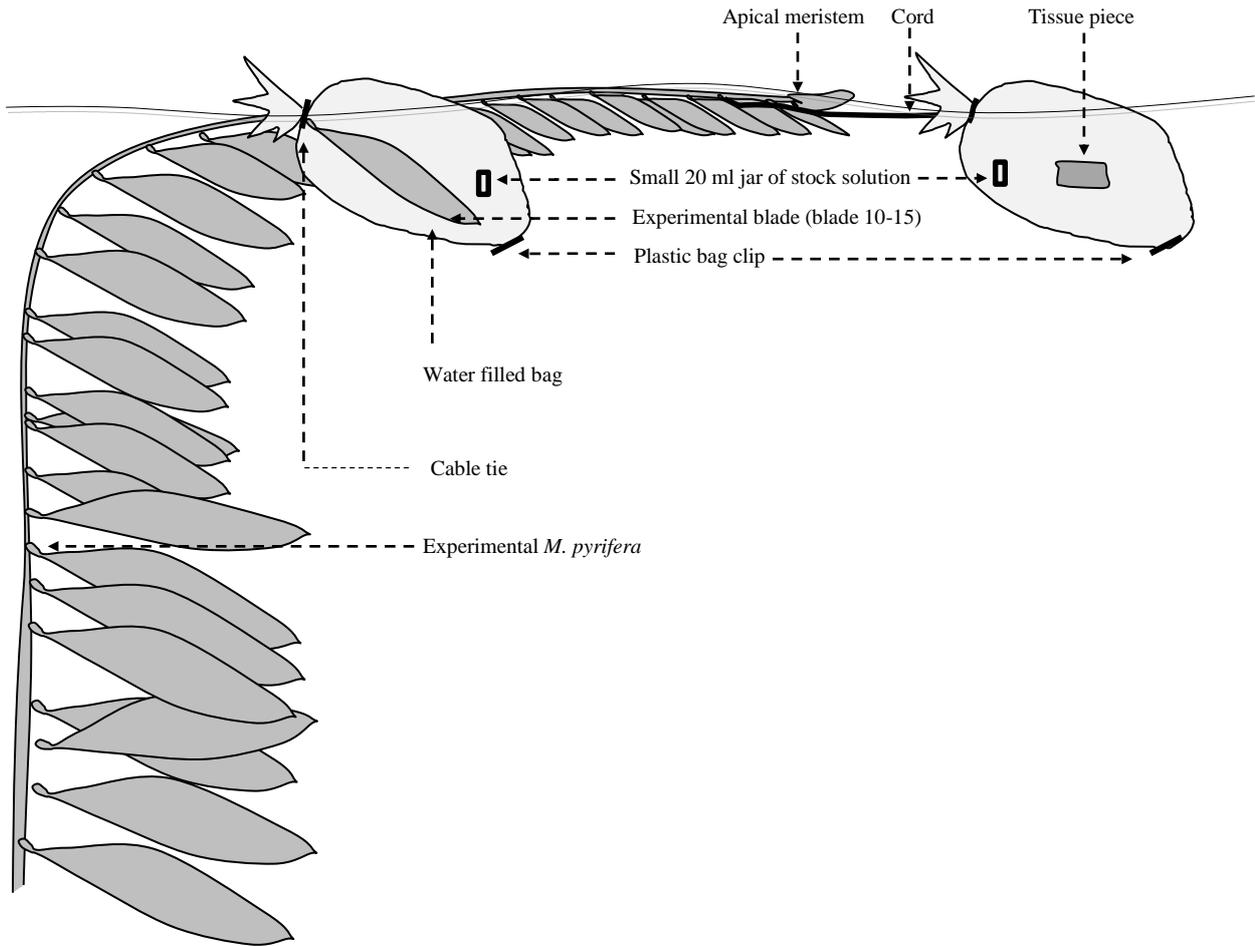
Figure 2.3: Experimental layout of *M. pyrifera in situ* nitrogen (ammonium and nitrate) uptake experiments.

(2.3) Results

(2.3.1) Preliminary time course experiments

Preliminary time course experiments revealed that blades would take up ammonium whilst in a transparent polyethylene bag (Figure 2.4) and thus *in situ* uptake experiments were performed. Uptake of ammonium was approximately linear over the 100 minute incubation period (Figure 2.4). Time course data is shown for ammonium only as the processing of nitrate samples rendered erroneous data. The cause of this is unknown.

(2.3.2) *In situ* ammonium and nitrate uptake by *M. pyrifera*

(2.3.2.1) Experimental conditions

The summer seawater temperature was 16.7°C whilst the spring seawater temperature was 12.0°C. *In situ* light levels were variable throughout both experimental periods. The summer light levels were 595 ± 57 μmol m^{-2} s^{-1} and the spring light levels were 851 ± 54 μmol m^{-2} s^{-1}.

(2.3.2.2) Control bag uptake

The change in ammonium and nitrate concentration within the control bags (no *M. pyrifera* present) was variable. In some instances the initial seawater concentration within the bag was ≤ 1 μM above the final concentration. In other instances the final concentration in the bag was ≤ 1 μM above the initial concentration. Additionally, on some occasions the final concentration exceeded the initial concentration to a degree that was unrealistic (possible explanations for this are discussed in section 2.4.5). Given the variability in the change in concentration of the control bags, it is not possible to give an estimate of percentage inorganic nitrogen lost due to photosynthetic organisms present in the unfiltered water. It will be assumed that the loss of nitrogen from the bags due to photosynthetic organisms in the water is minimal as was observed in the comparable study by Gerard (1982a). It was thus inferred that a majority of ammonium and nitrate removed from the *M. pyrifera* bags was attributed to the attached *M. pyrifera* blade or blade piece.

(2.3.2.3) Ammonium uptake

In late spring and summer, *M. pyrifera* ammonium uptake increased linearly at the studied concentrations displaying rate-unsaturable uptake (Figure 2.5 a, b, Table 2.1). Regression analysis revealed that the seasonal slopes for both excised and attached tissue did not differ significantly ($F = 0.012$, (3, 37), p = 0.998). Consequently, the late spring and summer data for both tissue types were pooled and a regression analysis performed (Figure 2.6 a, b, Table 2.1). The pooled data revealed that there was a significant linear relationship between *M. pyrifera* uptake rate and ammonium substrate concentration (Figure 2.6 b, Table 2.1).

(2.3.2.4) Nitrate uptake

Nitrate data for the attached *M. pyrifera* blade in summer and *M. pyrifera* blade piece in spring was limited (due to the final seawater nitrate concentration in the bag being higher than the initial nitrate concentration on some occasions) and hence nitrate uptake by the different tissue types could not be compared by season. When the data for both tissue types were combined and analysed according to season, uptake of nitrate displayed evidence of being both rate-saturable and rate-unsaturable (Figure 2.7 a, b). In summer there was some evidence of rate-unsaturable uptake (Figure 2.7 a, Table 2.2). However, there was limited data and the possibility that uptake may also be rate-saturable initially ($V_{max} = 15.83$ µmol gdw^{-1}h^{-1}, $K_s = 33.96$ µM) and then at higher substrate concentrations there is the potential for uptake to increase linearly (Figure 2.7 a, Table 2.2). Likewise, spring uptake showed evidence of saturation initially ($V_{max} = 9.18$ µmol gdw^{-1}h^{-1}, $K_s = 8.44$ µM) and then at higher substrate concentrations uptake appeared to increase linearly (Figure 2.7 b, Table 2.2).

Because of limited data and the discontinuity of nitrate uptake, the seasonal uptake curves and slopes of the differing tissue types could not be compared. However, because the standard errors and the 95% confidence intervals for the kinetic parameters were overlapping for pooled tissue type data from both summer and spring (Table 2.2), it was assumed that the *M. pyrifera* nitrate uptake did not differ by season. Hence, data from summer and spring were pooled and data that showed evidence of saturation had the Michaelis-Menten model applied. Pooled data for both tissue types and seasons displayed evidence of rate-saturable uptake initially ($V_{max} = 31.67$ µmol gdw^{-1}h^{-1}, $K_s = 61.00$ µM) and then following this, nitrate uptake appeared to increase linearly (Figure 2.8, Table 2.2).
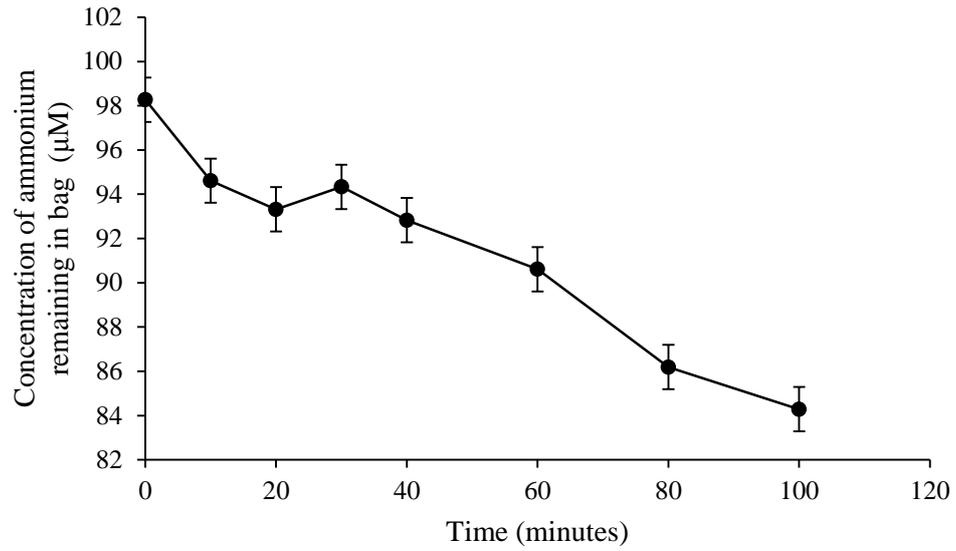
Figure 2.4: Depletion of ammonium from within a polyethylene bag due to uptake from a detached *M. pyrifera* blade. Data points are averages ± 1S.E, n = 3.
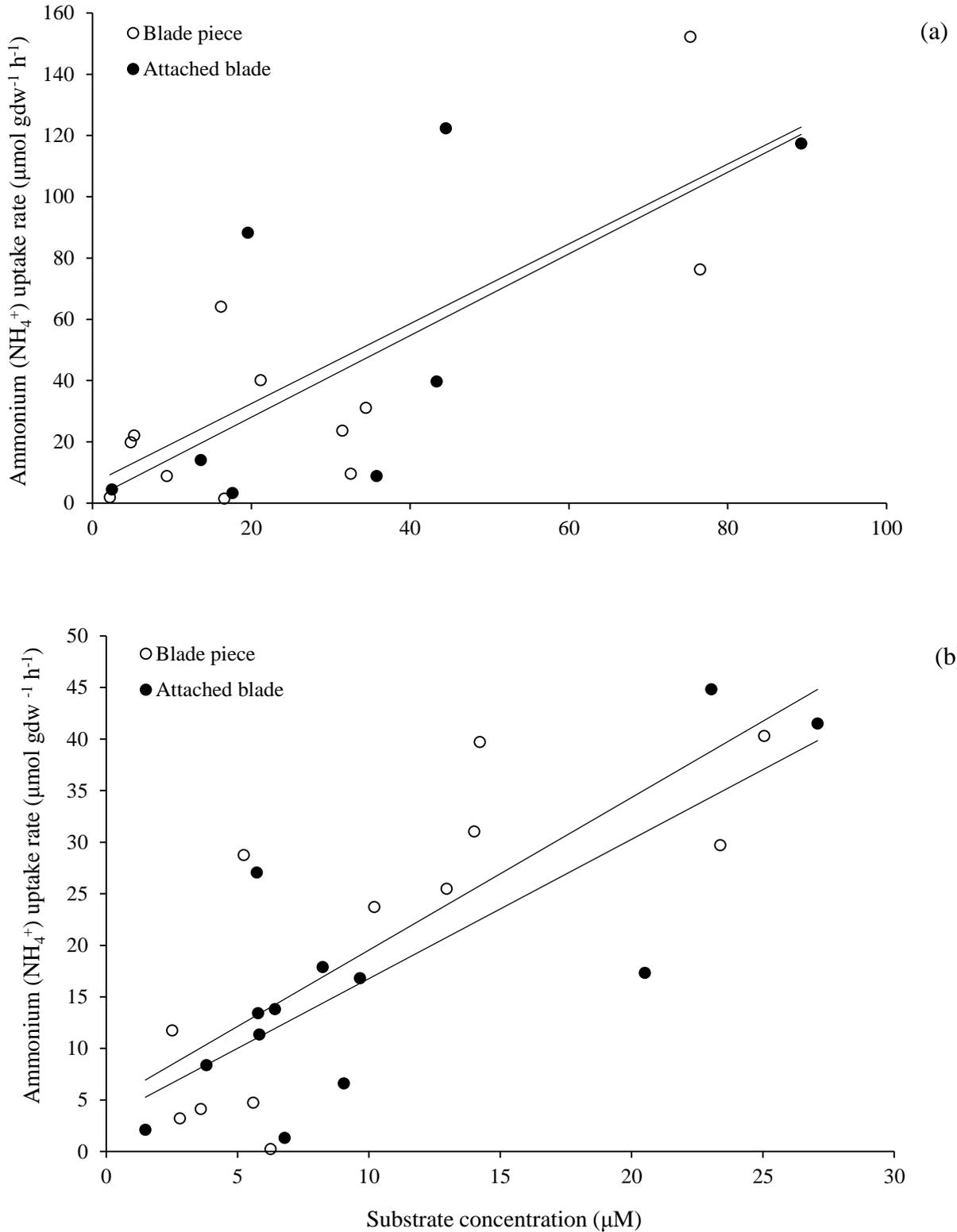
Figure 2.5: Ammonium uptake as a function of substrate concentration for *in situ* attached *M. pyrifera* blades and *M. pyrifera* blade pieces in summer (a) and spring (b), Golden Bay, Stewart Island, New Zealand.
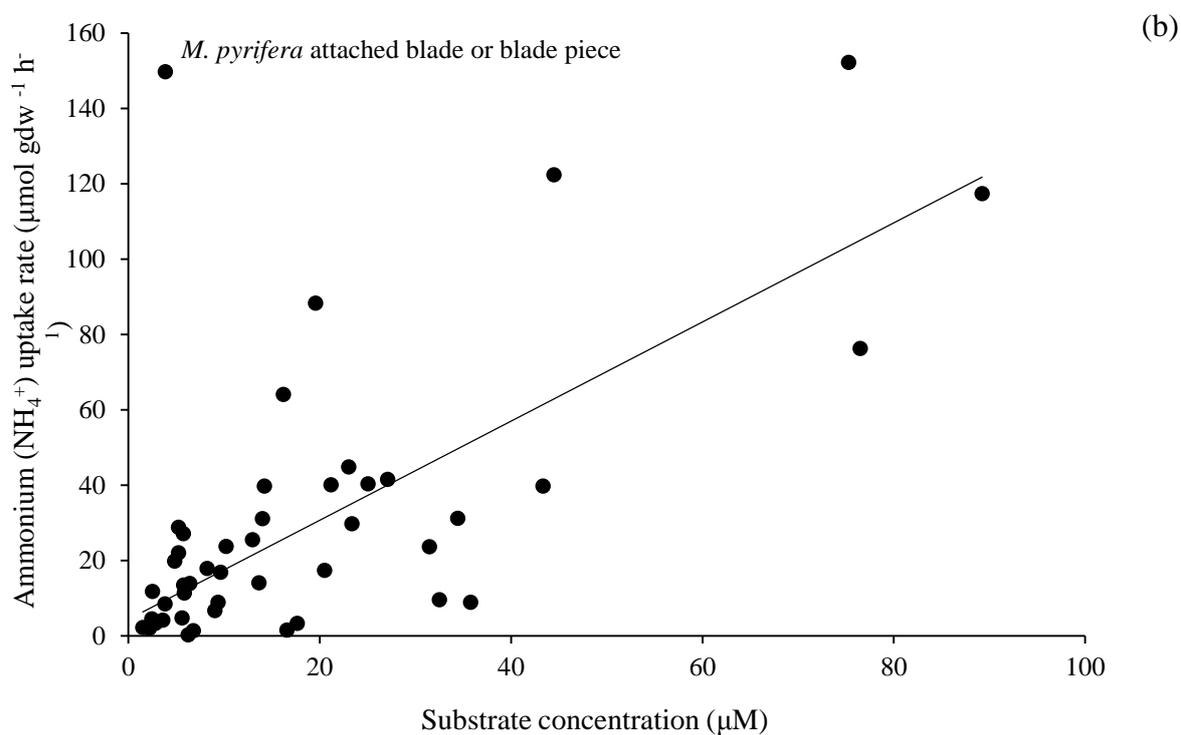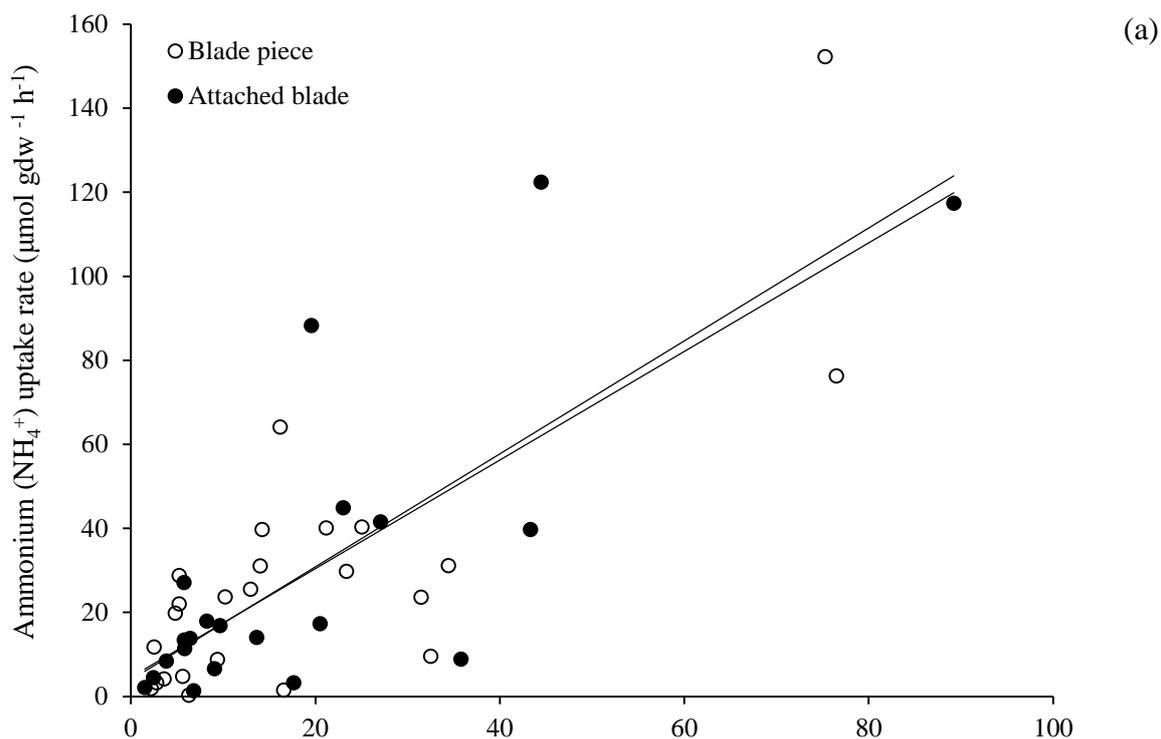
Figure 2.6: Ammonium uptake as a function of substrate concentration for pooled seasonal (summer and spring) *in situ* data for attached *M. pyrifera* blades and *M. pyrifera* blade pieces (a) and, ammonium uptake as a function of substrate concentration for pooled season (a) (summer and spring) and tissue type (attached *M. pyrifera* and *M. pyrifera* blade pieces) ... *situ* data for *M. pyrifera* (b) from Golden Bay, Stewart Island, New Zealand.
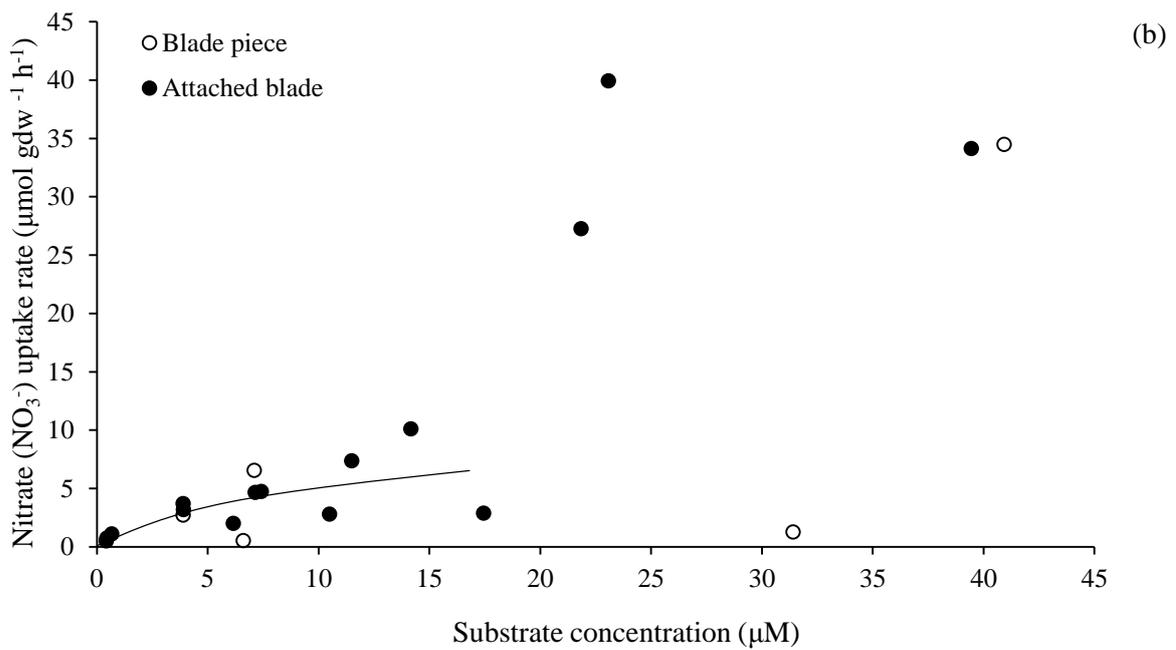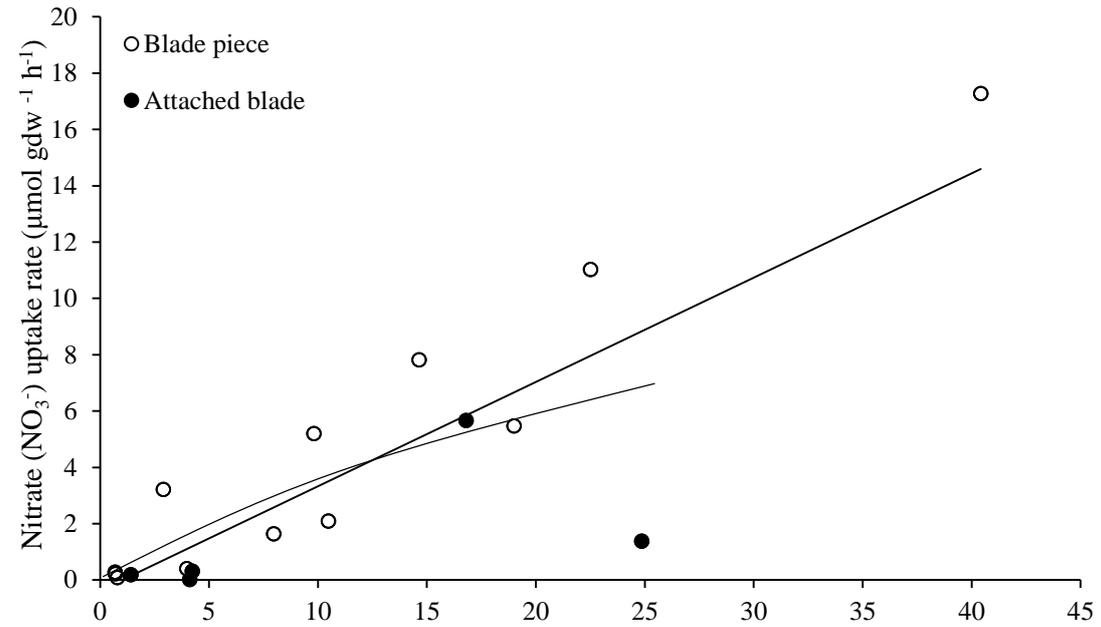
Figure 2.7: Nitrate uptake as a function of substrate concentration for *in situ* attached *M. pyrifera* blades and *M. pyrifera* blade pieces in summer (a) and spring (b), Golden Bay, Stewart Island, New Zealand.
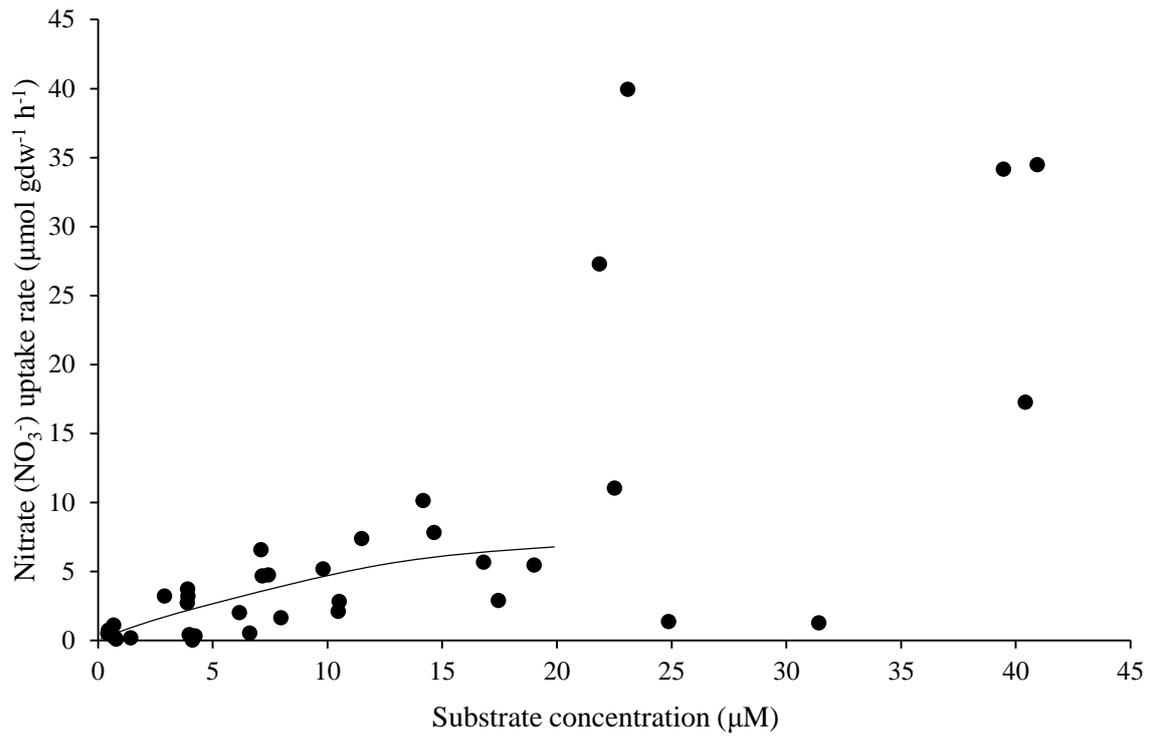
Figure 2.8: Nitrate uptake as a function of substrate concentration for pooled seasonal (summer and spring) and tissue type (attached *M. pyrifera* and *M. pyrifera* blade pieces) *in situ* data for *M. pyrifera* from Golden Bay, Stewart Island, New Zealand.

Table 2.1: Seasonal ammonium uptake kinetic parameters for different forms of *in situ M. pyrifera* tissue, Golden Bay, Stewart Island, New Zealand.

	NH$_4^+$				
	Regression equation	**Slope**	**r^2**	***p***	**n**
<u>Summer</u>					
Attached Blade	y = 1.304x + 6.426	1.304 ± 0.565	0.469	0.06	8
Blade Piece	y = 1.334x + 1.387	1.334 ± 0.333	0.615	0.0025	12
Attached blade + blade piece	y = 1.331x + 3.088	1.331 ± 0.282	0.551	0.0002	20
<u>Spring</u>					
Attached blade	y = 1.351x + 3.253	1.351 ± 0.307	0.637	0.001	13
Blade piece	y = 1.480x + 4.722	1.480 ± 0.379	0.603	0.002	12
Attached blade + blade piece	y = 1.413x + 3.96	1.413 ± 0.234	0.612	<0.0001	25
<u>Spring and summer</u>					
Attached blade	y= 1.344x+ 4.000	1.344 ± 0.256	0.5902	<0.0001	21
Blade piece	y = 1.292x + 4.611	1.292 ± 0.207	0.6369	<0.0001	24
Pooled seasonal data for both attached blade and blade piece	y = 1.317x + 4.318	1.317 ± 0.159	0.612	<0.0001	45

Parameters derived from linear regressions. Where appropriate, values are averages ± 1S.E.

Table 2.2: Seasonal nitrate uptake kinetic parameters for *in situ M. pyrifera* tissue, Golden Bay, Stewart Island, New Zealand.

	Regression equation	slope (α)	r^2	p	n	V_{max} (µmol gdw^{-1} h^{-1})	K_s (µM)	V_{max} 95% CI	K_s 95% CI
Summer									
Linear regression	y = 0.370x - 0.371	0.370±0.058	0.731	<0.0001	16				
Mechalis-Menton data●		0.466	0.527		16	15.83±16.55	33.96±62.40	-26.11, 57.77	0.0, 167.8
Spring									
Mechalis-Menton data†		1.088	0.412		15	9.18±5.638	8.44±10.55	-2.999, 21.36	0.0, 31.23
Spring and summer									
Mechalis-Menton data*		0.519	0.594		30	31.67±38.64	61.00±91.36	-4.907, 32.30	0.0, 64.94

Parameters derived from fitting a linear regression to data that displayed evidence of rate-unsaturable uptake and the Mechalis-Menton model to data that displayed evidence of rate-saturable uptake. There was insufficient data to enable a seasonal comparison of nitrate uptake by the different tissue types. Consequently, data from both tissue types was pooled and the Mechalis-Menton model applied to data that displayed evidence of rate-saturable uptake. Removal of data points where substrate concentration was above 25 µM is denoted by a ●. The removal of data points where substrate concentration was above 20 µM is denoted by a †. The removal of outliers in the analysis is denoted by an *. These data values were removed so the Mechalis-Menton model could be fitted to the data. Where appropriate, values are averages ± 1S.E.

(2.4) Discussion

(2.4.1) Uptake rate and relationship to substrate concentration

The uptake of nitrate by brown macroalgae often displays a rate-saturated response (Gerard 1982a; Wallentinus 1984; Thomas et al. 1985; Braga and Yoneshigue-Valentine 1996; Hurd et al. 1996; Torres et al. 2004; Phillips and Hurd 2004; Dean and Hurd 2007; Rees et al. 2007; Wang et al. 1914b). Saturable nitrate uptake for *M. pyrifera* has been observed both *in vitro* (Haines and Wheeler 1978; Kopczak 1994) and *in situ* (Gerard 1982a). Gerard (1982a) analysed the *in situ* nitrogen uptake kinetics of *M. pyrifera* using the bag approach utilised in this study and was able to determine that *M. pyrifera* from California exhibited rate-saturable nitrate uptake which is somewhat in agreement with this study. In this study *M. pyrifera* nitrate uptake appeared to show uptake discontinuity where uptake initially was saturated ($V_{max} = 31.67$ μmol $gdw^{-1}h^{-1}$, $K_s = 61.00$ μM) and then increased linearly thereafter. Deviation away from typical hyperbolic uptake has been observed for ammonium uptake by *M. pyrifera* (Haines and Wheeler 1978) and other brown and red algae (D'Elia and DeBoer 1978; Thomas et al. 1985). Nitrate uptake discontinuity has not been observed for *M. pyrifera*. Because *M. pyrifera* displayed rate-saturable uptake kinetics at low nitrate concentrations, it is likely that uptake is via active transport (Harrison and Hurd 2001) and following this some other mechanism occurs. The latter mechanism could be diffusion via ion channels resulting in a linear relationship between substrate concentration and uptake rate or another uptake mechanism could be operating which will produce a second higher estimate of V_{max} and K_s. However, it cannot be guaranteed that an additional mechanism is operating as there are limited data values present at substrate concentrations above ~20 μM.

Rate-unsaturable (linear) uptake of ammonium has been reported for some brown seaweeds including *Ecklonia maxima* (Probyn and McQuaid 1985), *Laminaria groenlandica* (Harrison et al. 1986), *Xiphophora chondrophylla* (Taylor et al. 1998), *Scytothamnus australis* and *Xiphophora gladiata* (Phillips and Hurd 2004). In this study, ammonium uptake by *M. pyrifera* did not saturate at the highest studied concentration (~80 μM) but instead increased linearly. This result differs from studies that have documented ammonium uptake by *M. pyrifera in vitro*. *In vitro* experiments with *M. pyrifera* have displayed rate-saturable uptake kinetics for ammonium (Haines and Wheeler 1978; Wheeler 1979) although Haines and Wheeler (1978) demonstrated that there was the potential for *M. pyrifera* to exhibit two uptake mechanisms

with ammonium saturating at lower concentrations and then increasing linearly at higher concentrations. However, it is of limited value to compare the results of this *in situ* study with the results of these *in vitro* experiments because of the significant difference in experimental conditions; this study was conducted in the field under natural light and water movements whilst the studies by Haines and Wheeler (1978) and Wheeler (1979) were conducted under controlled laboratory conditions. This is the first study to document rate-unsaturable ammonium uptake by *M. pyrifera in situ* and because uptake of ammonium did not saturate with increasing substrate concentration, it is likely that during late spring and summer uptake of ammonium by Stewart Island *M. pyrifera* is by passive diffusion.

(2.4.2) Analysis of kinetic parameters

M. pyrifera ammonium uptake was faster than nitrate uptake for both the attached *M. pyrifera* blades and blade pieces. Comparing the slopes of the ammonium and nitrate uptake revealed that uptake of ammonium by *M. pyrifera* was 2.5 times faster than for nitrate. A faster ammonium uptake rate is in agreement with other studies that have documented the ammonium and nitrate uptake kinetics of brown seaweeds (Wallentinus 1984; Thomas et al. 1985; Braga and Yoneshigue-Valentine 1996; Perderson and Borum 1997; Korb and Gerard 2000; Phillips and Hurd 2004; Torres et al. 2004) including *M. pyrifera* (Haines and Wheeler 1978). A faster ammonium uptake rate can be attributed to the capacity for surge uptake of ammonium by macroalgae and because of the reduced energy requirements for ammonium uptake (Harrison and Hurd 2001).

(2.4.3) The effect of tissue excision on uptake rate

It was hypothesised that tissue excision would affect the nitrogen uptake rates of *M. pyrifera* because of the physical damage that cutting has on the studied individual (Wheeler 1979). However, this study revealed that excising the *M. pyrifera* tissue had no significant effect on the *in situ* uptake of ammonium. This result differs with the results documented in Wheeler (1979) where it was found that *M. pyrifera* apical blade or mature blade tissue excision can reduce methylamine (an ammonium analogue) uptake by 30-80% (depending on tissue type). Differing results in this study could be explained by the different chemical properties of

methylamine relative to NH$_4$Cl or possibly the recovery time allocated to the excised tissue. After tissue excision, Wheeler (1979) allowed a tissue recovery time of six hours before the *in vitro* uptake experiments were undertaken. In this study a recovery time of at least eighteen hours was allocated to the excised *M. pyrifera* blade pieces. The differing recovery period could explain why Wheeler (1979) found that excising *M. pyrifera* tissue affected methylamine uptake whereas in this study excising *M. pyrifera* tissue did not significantly affect ammonium uptake. This result has important applications for future nutrient uptake experiments where excised macroalgal tissue is used. This research suggests that excised macroalgal tissue needs to be given an adequate recovery period if accurate results are to be obtained.

(2.4.4) Translocation and nutrient uptake

Large complex Laminariales (like *M. pyrifera*) are capable of long distant transport of substances to distant storage sinks (Parker 1966; Schmitz and Srivastava 1979a; Schmitz and Srivastava 1979b; Manley 1981; Raven 2003, Hepburn et al. 2012). It was thus hypothesised that tissue excision would affect the nitrogen uptake rates of *M. pyrifera* by removing the potential for the translocation of nitrogen. However, as outlined in section 2.4.3 tissue excision did not significantly affect the nitrogen uptake rates of *M. pyrifera*. It can therefore be deduced that either no translocation was occurring or the extent of translocation by attached *M. pyrifera* was insufficient to allow for a significant effect on *M. pyrifera* nitrogen uptake to be observed.

A likely explanation for lack of difference in uptake rates between excised and attached tissue and hence lack of translocation is the short incubation period duration. The *M. pyrifera* blades were only exposed to the nutrient solution for a one hour time period (an incubation period of one hour or less is often used in *in vitro* multiple flask experiments (Thomas et al. 1985; Rosenberg and Ramus 1984; Harrison et al. 1986; Phillips and Hurd 2004; Rees et al. 2007; Kregting et al. 2008; Wang et al. 2014b)). It is possible that one hour is an insufficient time period for translocation to be measured. For *M. pyrifera*, Hepburn et al. (2012) found that a time period of four hours was of sufficient duration to observe the transport of a ^{15}N enriched ammonium solution from a *M. pyrifera* blade to other regions of the individual. Because it is unlikely that insufficient translocation is occurring during this short *in situ* uptake experiment, the absence of the capacity for translocation in *in vitro* uptake experiments is unlikely to be a significant factor affecting the measured nutrient uptake rates. Consequently, *in vitro* uptake

experiments using cut pieces of tissue could still yield important and useful information on the nutrient uptake rates of *M. pyrifera* and potentially other macroalgae.

(2.4.5) Difficulties with the *in situ* method

Although conducting *in situ* experiments provides a novel and interesting way to estimate macroalgal nutrient uptake kinetics, there are difficulties associated with this method. In this study one particular problem that occurred was the failure of some bagged *M. pyrifera* to exhibit uptake or the final water sample taken having a higher nitrogen concentration than the initial water sample concentration. Theoretically this should not occur as there should be nitrogen uptake by the *M. pyrifera* blade or blade piece. Possible reasons for these problems could be inadequate *in situ* water movement, excretion of ammonium and/or nitrate by the *M. pyrifera* tissue, bacterial activity and poor initial mixing.

Inadequate *in situ* water movement could have enabled the formation of boundary layers. Inadequate water movement and the resulting boundary layer formation can inhibit or reduce *M. pyrifera* nutrient uptake (Wheeler 1980; Gerard 1982b; Hurd et al. 1996). Notably, during some experimental days, water movement *in situ* was minimal as clement weather and conditions are required for conducting *in situ* experiments. However, Gerard (1982b) quantified *in situ* water movement at the *M. pyrifera* blade surface and found that even under very calm conditions, water movement at the blade surface will be sufficient to allow for saturable nitrate uptake. So although there is the potential for boundary layer formation to inhibit *M. pyrifera* nitrogen uptake, it is unlikely that boundary layer formation is the reason for the failure of some bagged blades to exhibit uptake.

Whilst the *in situ* experiments were being conducted, the *M. pyrifera* tissues could have released sequestered nitrogen as exudates (*sensu* De Burgh and Fankboner 1978; Abdullah and Fredriksen 2004; Salaün et al. 2012) and this could have resulted in higher than expected concentrations of ammonium/nitrate in the bags. It is also possible that bacterial activity in the water or on the *M. pyrifera* tissue could have accounted for some of the ammonium or nitrate uptake (Wheeler 1979). However, poor initial mixing could be a more likely reason for some bags exhibiting higher final nitrogen concentrations as opposed to initial nitrogen concentrations.

After the summer *in situ* experiments were conducted, the bag mixing period of fifteen seconds was changed to thirty seconds with the aim of improving mixing and reducing the number of instances where the final nitrogen concentration in the bag was higher than the initial concentration. Additional mixing did appear to help reduce the prevalence of this problem but the problem of higher final nitrogen concentrations remained at times. Future *in situ* experiments using the bag approach should mix the bag for at least sixty seconds or the experimenters should conduct preliminary experiments to determine an adequate mixing period.

(2.5) Conclusion

In situ M. pyrifera exhibits faster uptake for ammonium than nitrate. *M. pyrifera* displayed rate-unsaturable ammonium uptake with uptake increasing linearly at the studied concentrations (~80 μM). Nitrate uptake displayed evidence of both rate-saturable and rate-unsaturable uptake indicating the potential for two uptake mechanisms to be operating. Excision of the *M. pyrifera* blade did not significantly affect the uptake of ammonium. It is possible that a long tissue recovery period allowed the excised tissue to recover to a degree that ammonium uptake was unimpaired. It is also likely that a short incubation period (one hour) meant that absence of the capacity for translocation was not a significant factor influencing uptake over the incubation period. Future nutrient uptake experiments should ensure an adequate recovery period is allocated to excised macroalgal tissue to enhance the accuracy of nutrient uptake parameters.

Chapter 3   Integrated multi-trophic aquaculture (IMTA) trials with the macroalga *Macrocystis pyrifera*, **salmon (***Oncorhynchus tshawytscha***) and mussel (***Perna canaliculus***) in Big Glory Bay, Stewart Island, New Zealand.**

(3.1) Introduction

(3.1.1) New Zealand aquaculture status

The New Zealand aquaculture industry is growing and the New Zealand Government's aquaculture strategy outlines plans for a $1 billion aquaculture industry by 2025 (Burrell and Meehan 2006). Currently, the primary aquaculture species in New Zealand are green-lipped (Greenshell™) mussels (*Perna canaliculus*), Chinook (king) salmon (*Oncorhynchus tshawytscha*) and Pacific oysters (*Crassostrea gigas*) (Burrell and Meehan 2006). However, the government aquaculture strategy documents the need for new and innovative aquaculture species (Burrell and Meehan 2006). Additionally, the need for sustainability has also been highlighted (Burrell and Meehan 2006). Macroalgae have the potential to contribute to both the economic growth and sustainability objectives. Economically, macroalgae have potential as they can be used in alginate, pharmaceutical, carrageenan and fertiliser manufacture and macroalgal products are consumed as food and health supplements by both humans and animals (Radmer 1996; Zemke-White and Ohno 1999; Smit 2004; Gutierrez et al. 2006; Flores-Aguilar et al. 2007 FAO 2014). With regards to sustainability, macroalgae can be used to mitigate nutrient enrichment through their bioextraction of dissolved inorganic nutrients (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2009).

(3.1.2.) Remediation of aquaculture-induced nutrient enrichment

Nutrient enrichment is commonly associated with the aquaculture of higher trophic level organisms such as fish, and finfish aquaculture-induced nutrient enrichment is a consequence of nutrient rich fish feed and fish excretory matter (Silvert 1992; Chen et al. 2003; Islam 2005; Mente et al. 2006; Fernandes et al. 2007; Wang et al. 2014a). Consequences of nutrient enrichment can include harmful algal blooms (HABs) and the development of anoxic benthic conditions (Silvert 1992; Hallegraeff 1993; Van Dolah 2000). These consequences can negatively affect ecosystem function (Wu et al. 1994; Meyer-Reil and Köster 2000; Deegan et al. 2002), human health and commercial activities (Shumway 1990; Hallegraeff 1993;

Galloway et al. 2003) which can be barriers to the expansion of aquaculture and major sources of public aquaculture criticism (Neori 2008). To gain public support for aquaculture expansion, these potential consequences need to be remediated. The use of macroalgae in an integrated multi-trophic aquaculture (IMTA) approach is one method that can be used to alleviate aquaculture-induced nutrient enrichment (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2009).

IMTA is an aquaculture technique which involves the co-culture of organisms of differing trophic levels (usually finfish with bivalves and/or macroalgae) (Chopin et al. 2001; Troell et al. 2009). Typically, an IMTA approach utilises the ability of macroalgae to take up and store dissolved nutrients originating from the farming process, thereby reducing the level of nutrient enrichment and the risk of consequences associated with increased nutrient concentrations (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2009). IMTA has been researched and applied in North America, Asia, The Middle East, South America and Europe (Chopin et al. 2001; Bostock et al. 2010). In China, Canada, The United Kingdom, Israel and South Africa, IMTA has been put into commercial practice (Neori et al. 2004; Troell et al. 2009; Bostock et al. 2010). Despite some success internationally, IMTA has received relatively little attention in New Zealand. However, there have been scientific trials of macroalgae (NIWA 2007) and sea cucumbers (MacTavish et al. 2012). Further IMTA research in New Zealand will be important if New Zealand is to effectively utilise IMTA to reach growth goals in a sustainable and profitable manner.

(3.1.3) Open water IMTA research approach

When determining the suitability of a macroalgal species for use in open water IMTA, it is important to have information on the seawater chemistry of the proposed IMTA site as well as information on the ecophysiology of the selected macroalgae when subjected to a potential IMTA site (Wu et al. 1994; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Abreu et al. 2009; Sanderson et al. 2012; Wang et al. 2014a). The analysis of seawater chemistry at a potential growing site, specifically concentrations of ammonium and phosphate, can give insight into the extent to which the fish farm is providing nutrients which co-cultured macroalgae could utilise (Wu et al. 1994; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Sanderson et al. 2012; Abreu et al. 2009; Wang et al. 2014b). Physiological

parameters of interest include macroalgal growth rate data, carbon and nitrogen status (percentage carbon, percentage nitrogen and carbon:nitrogen ratio (C:N)), isotopic signatures (δ^{15}N) and soluble nitrogen (ammonium and nitrate) and pigment concentrations. Assessing growth rates can indicate how nitrogen supplementation from a fish farm can support macroalgal growth especially during periods when growth is nitrogen limited (Wu et al. 1994; Troell et al. 1997; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Abreu et al. 2009; Sanderson et al. 2012; Wang et al. 2014a). Analysing percentage nitrogen and C:N in the macroalgal tissue may reveal the extent to which a macroalgal species is taking up aquaculture derived nitrogen as changes in nitrogen provision can influence these parameters (Wu et al. 1994; Troell et al. 1997; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Abreu et al. 2009; Sanderson et al. 2012; Wang et al. 2014b). Macroalgal δ^{15}N signatures can give insight into the origin and extent of sequestered nitrogen as the δ^{15}N signatures move towards the δ^{15}N signature of the fish food or excretory matter (Vizzini and Mazzola 2004; García-Sanz et al. 2010; García-Sanz et al. 2011; Wang et al. 2014a). Analysing macroalgal soluble nitrogen and pigment concentrations could also produce useful data on the extent of macroalgal nitrogen sequestration as nitrogen can be stored intracellularly in these pools or as pigments (Bird et al. 1982; Rosenberg and Ramus 1982; Shivji 1985; Stengel and Dring 1998; Harrison and Hurd 2001; Kim et al. 2007; Ribeiro et al. 2013).

(3.1.4) *Macrocystis pyrifera* and its potential for IMTA in New Zealand

Macrocystis pyrifera is a macroalgal species that has the potential to be used in IMTA in New Zealand. Internationally, *M. pyrifera* has been identified as a suitable IMTA species due to its physiological characteristics (growth rate and nutrient uptake ability) (Buschmann et al. 2008; Hadley et al. 2015) and economic potential particularly in aquaculture feed, organic fertilizer, food products and health supplement manufacture (Gutierrez et al. 2006; Flores-Aguilar et al. 2007; Correa et al. in press). Additionally, *M. pyrifera* has recently been added to the quota management system in New Zealand and interest in harvest of wild populations may increase. This has generated some concern because *M. pyrifera* is an important ecosystem engineer (*sensu* Jones et al. 1994; Jones et al. 1997) and globally *M. pyrifera* populations are threatened by fishing pressure, invasive species, extreme weather events, anthropogenic disturbance and climate change (Dayton and Tegner 1984; Seymour et al. 1989; Tegner and Dayton 2000; Foster and Schiel 2010; Krumhansl et al. 2011; Harley et al. 2012). Also of concern is that

harvesting *M. pyrifera* tissue can negatively affect *M. pyrifera* reproduction (Geange 2014) and alter the composition of understory algal communities (Santelices and Ojeda 1984). Consequently, harvesting from wild populations may not be in the interest of the marine ecosystem. It would be preferable to farm *M. pyrifera* in an IMTA approach and receive the benefit of nutrient removal and potentially habitat creation as well as an economically useful product.

(3.1.5) Rationale for site selection

The study site for IMTA trials using *M. pyrifera* is Big Glory Bay, Paterson Inlet, Stewart Island, New Zealand. Big Glory Bay provides an excellent opportunity to study the potential for IMTA using *M. pyrifera* because within the bay there are both salmon and bivalve (mussel and oyster) farms in close proximity of one another. Additionally, historically the aquaculture operations within the bay have had problems with HABs causing fish fatalities and bivalve toxicity (Chang et al. 1990; MacKenzie 1991). If IMTA could be successfully utilised in Big Glory Bay there is the potential to ameliorate the risk of HABs. Additionally, the waters of Stewart Island are clear and have low summer nitrogen concentrations (Hepburn and Hurd 2007; Stephens and Hepburn 2014). Nitrogen limitation during this time can impede *M. pyrifera* growth (Hepburn et al. 2007; Stephens and Hepburn 2014) and hence nitrogen supplementation from the aquaculture operations in Big Glory Bay could allow for improved *M. pyrifera* growth during this time.

(3.1.6) Aims and hypotheses

Given the problems associated with aquaculture-induced nutrient enrichment and the potential for *M. pyrifera* to be used in IMTA in New Zealand, this study assessed the suitability of *M. pyrifera* as an IMTA species for use in Big Glory Bay, Stewart Island, New Zealand. Specifically, this study determined how the presence of the Kiwa 1 salmon farm and a mussel farm affected the summer and autumn growth rate, carbon and nitrogen status, $\delta^{15}N$ signatures and soluble nitrogen and pigment concentrations of experimentally grown *M. pyrifera*. Because summer *M. pyrifera* growth can be nitrogen limited (Zimmerman and Kremer 1984; van Tussenbroek 1989; Brown et al. 1997; Hepburn et al. 2007; Stephens and Hepburn 2014) and

fish and bivalves can supply nitrogen for use by macroalgae, it was predicted that *M. pyrifera* growth, percentage nitrogen, and soluble ammonium concentration would be greater at the salmon and mussel sites compared to the control site particularly during the summer low-nitrogen growing period. The influence of nitrogen provision from the salmon and mussel farm on these parameters was expected to decline during autumn due to increased naturally occurring seawater nitrogen concentrations negating the effect of nitrogen provision from the salmon and mussel farms and light having a greater influence on growth as days get shorter (Hepburn et al. 2007). It was also anticipated that the $\delta^{15}N$ signature of *M. pyrifera* from the salmon and mussel site would be heavier than the $\delta^{15}N$ signature of *M. pyrifera* from the control site. Finally, higher pigment concentrations were expected in *M. pyrifera* from the salmon and mussel sites because macroalgae typically have higher pigment concentrations when nitrogen replete (Bird et al. 1982; Rosenberg and Ramus 1982; Shivji 1985; Stengel and Dring 1998; Kim et al. 2007; Ribeiro et al. 2013).

(3.2) Methods

(3.2.1) Experimental location

The experiment was conducted at Big Glory Bay (46°58'53.68"S, 168° 7'14.06"E) and Glory Cove (46°58'5.13"S, 168° 9'43.92"E), Stewart Island, New Zealand (Figure 3.1). Within Big Glory Bay there are two finfish farms, one farm rearing adult *O. tshawytscha* (Kiwa 1) and another raring juvenile *O. tshawytscha* (Kiwa 2). Approximately 2,400 tonnes of salmon are harvested annually (P. Nicholson, personal communication, February 2, 2015). There are numerous mussel (*P. canaliculus*) and oyster (*Tiostrea chilensis*) farms distributed throughout Big Glory Bay. Glory Cove is a neighboring bay with no aquaculture present and like Big Glory Bay, its catchment is native bush.

(3.2.2) Big Glory Bay and Glory Cove water chemistry

To gain an insight into the water chemistry surrounding the Kiwa 1 salmon farm and Glory Cove control site, surface water samples were taken in December 2013 and May and April 2014. During summer (December), thirteen 10 ml water samples (10% HCl washed containers) were taken from directly next to the farm (at different locations) and nine 10 ml water samples were taken at the Glory Cove control site at three tidal phases, slack tide, mid-high tide and mid-low tide. Control site water samples were taken haphazardly whilst travelling around the bay in a 7.3 metre NAIAD boat. Water samples were not taken at the mussel site in summer. In autumn (April and May), five 10 ml water samples were taken from just outside the salmon farm (the location of the experimentally grown *M. pyrifera*, ~25 metres from the salmon farm edge) and from within Glory Cove. After all samples were collected they were filtered (Whatman™ GF/C) and frozen for later analysis of ammonium, nitrate and phosphate. After defrosting the samples, ammonium, nitrate and phosphate in each sample was determined using a Quickchem 8500® automated ion analyser (Lachat Instruments, Milwaukee, USA) according to standard methods outlined in Strickland and Parsons (1968) and Lachat Instruments (2008). Ammonium concentration in the seawater samples was estimated using the indophenol-blue method. Nitrate concentration in the seawater samples was estimated by reduction with a copper-coated cadmium column. Phosphate (orthophosphate ion PO_4^{3-}) was determined by the reaction of ammonium molybdate and antimony potassium tartrate under acidic conditions, reduced with ascorbic acid forming a blue complex which is measured spectrophotometrically.

(3.2.3) *M. pyrifera* **experimental growing procedure and data collection**

In January and April 2013, juvenile *M. pyrifera* were collected using SCUBA at a depth of 2-3 meters from Horseshoe Bay (non-aquaculture site), Stewart Island, New Zealand (46°52'39.51"S, 168°8'44.34"E). After collection, each *M. pyrifera* individual had its wet weight determined (mean = 48.3 g, SE = 2.73 g) before being assigned to an experimental growing frame. Growing frames (Figure 3.2) were constructed from a one metre long piece of polyethylene pipe (35 mm diameter) with two 2.5 metre lengths of rope fed through the pipe. The ends of each rope were tied together making two loops. One of the loops had a mesh sack (onion sack) wrapped around it creating a pouch. This pouch was sewn up and split into four sections using cable ties. Each section had a juvenile *M. pyrifera* added to it. Cable ties were then used to fasten the juvenile *M. pyrifera* to the rope and mesh sack. Each growing frame and section within the growing pouch was labeled so individual *M. pyrifera* could be later identified. To quantify light, a growing frame from each site had a HOBO light logger (Onset, Massachusetts USA) fixed to the frame with a cable tie.

The growing frames (nine total, three per site in summer and twelve total, four per site in autumn) were then transported to the growing sites by boat. There were three growing sites categorised as salmon, mussel and control. At the salmon site (46°58'53.68"S, 168°7'14.06"E) (Figure 3.1) the *M. pyrifera* were grown in close proximity (~25 metres) to the Kiwa 1 salmon farm. Permission was given by farm management to deploy the growing frames on two salmon farm mooring lines. The mussel site was a mussel line close to the entrance of Big Glory Bay (46°58'21.26"S, 168°8'23.59"E) (Figure 3.1) (~2 km away from the salmon farm). At the mussel site, the growing frames were deployed three metres apart along a surface running mussel farm support line. At the control site, the growing frames were deployed in three (summer) or four (autumn) haphazardly selected sub-sites in neighboring Glory Cove (~5.5 km from the salmon farm) (46°58'5.13"S, 168°9'43.92"E) (Figure 3.1). At each site, the frames were deployed at a depth of two metres and were held in place with a 17 kg concrete mooring.

Following a growing period of 26 days in summer and 28 days in autumn, the growing frames were removed and the *M. pyrifera* individuals were harvested. After harvesting, each *M. pyrifera* individual was re-weighed to determine its final wet weight. The tissue from the apical meristem region of each individual was removed, rinsed with fresh water (to remove attached epifauna), placed in labeled bags and frozen for later analysis.

(3.2.4) Analysis of *M. pyrifera* growth rate

After the growing periods, the relative growth rate (RGR) of each *M. pyrifera* individual was determined using the equation: $RGR = (\ln W_2 - \ln W_1)/\Delta t$ (Abreu et al. 2009; Sanderson et al. 2012). W_1 is the wet weight (g) of the *M. pyrifera* individual at the time of growing frame deployment, W_2 is the wet weight of the *M. pyrifera* individual at the time of harvest, Δt is the elapsed time in days. The RGR of all *M. pyrifera* from a growing frame were averaged to create an average growth rate for each frame and statistical analysis was applied (growing frames were treated as replicates), n = 3 summer, n = 4 in autumn.

(3.2.5) *M. pyrifera* nitrogen status and $\delta^{15}N$ isotopic composition

Percentage nitrogen, percentage carbon, carbon:nitrogen (C:N), and stable isotope $^{15}N{:}^{14}N$ were determined for tissue harvested from the *M. pyrifera* grown at the different sites. Tissue samples from five haphazardly selected individuals (n = 5 in summer and autumn) from each site were dried at 50 ºC for seven days. Dried tissue samples were individually ground into a powder with a mortar and pestle. The mortar and pestle were carefully washed with acetone and MilliQ™ high purity water (18.2 MΩ·cm at 25 °C) and dried between samples to avoid contamination. Ground samples were then stored in sealed eppendorf (2 ml) tubes prior to determination of percentage nitrogen and carbon, C:N ratio and $^{15}N{:}^{14}N$. Determination of these parameters was performed by The Department of Chemistry at the University of Otago. Samples (1.2 mg ± 0.2 mg) of the *M. pyrifera* tissue were added to tin capsules and combusted via a Carlo-Erba® NC2500 elemental analyser (CE Instruments, Milan) interfaced to a Europa Scientific® "20/20 Hydra" (Europa Scientific, UK) isotope ratio mass spectrometer. Outputs from this procedure provided the percentage nitrogen and carbon, C:N ratio and $^{15}N{:}^{14}N$ of the *M. pyrifera* tissue.

(3.2.6) Salmon pellet and mussel tissue $\delta^{15}N$ determination

The $\delta^{15}N$ values of salmon pellets and green-lipped mussel tissue were determined because they are important parameters in a two source isotope mixing model (see section 3.2.9). To determine the $^{15}N{:}^{14}N$ of salmon pellets, twenty pellets were collected from the salmon farm

feed store. These pellets were dried at 50 °C for seven days. Five samples of salmon pellets were prepared with each sample consisting of four pellets that were ground using a mortar and pestle. Each sample was then placed in a labeled eppendorf 2 ml tube (n = 5). To determine the ^{15}N:^{14}N of green-lipped mussel waste, twenty mussels were harvested and removed from their shell. The kidney and pericardial glands are where bivalve waste is stored although bivalves also excrete nitrogenous waste across their tissue surfaces (Gosling 2003). As such, whole green-lipped mussels were individually dried at 50 °C for seven days. Five samples of mussel tissue were prepared consisting of tissue pieces from four mussels. The mussel tissue pieces were then ground with a mortar and pestle. Each sample was then placed in a labeled eppendorf 2 ml tube (n = 5). The dried samples of salmon pellets and mussel tissue were then taken to The Department of Chemistry at the University of Otago for δ^{15}N analysis. Samples (0.8 mg ±0.1 mg) were analysed according to methods described in section 3.2.5.

(3.2.7) Determination of soluble tissue pools

Soluble tissue ammonium and nitrate concentrations were determined using a boiling water extraction (Hurd et al. 1996). Tissue portions were cut from the apical meristem region of five *M. pyrifera* individuals from each site. The tissue was patted dry with a paper towel and weighed (~0.5 g) before being added to labeled 50 ml boiling tubes (10% HCl washed) with 40 ml of high purity water. The boiling tubes were then added to 500 ml beakers half filled with boiling water and the boiling tubes were brought to the boil and boiled for 20 minutes. Tissue portions were boiled once as Hurd et al. (1996) found that a single boiling extraction was sufficient. Following the boiling period, the boiling tubes were allowed to cool and 10 ml aliquots were filtered (Whatman™GF/C) into labeled 10 ml plastic tubes (10% HCl washed) and frozen for later determination of ammonium and nitrate. Following the defrosting of samples, ammonium and nitrate in each sample was determined using a Quickchem 8500® automated ion analyser (Lachat Instruments, Milwaukee, USA). Ammonium and nitrate were estimated using the methods outlined in section 3.2.2.

(3.2.8) Determination of pigments

Chlorophyll *a* and accessory pigments chlorophyll *c* and fucoxanthin were determined using methods adapted from Seely et al. (1972). Five haphazardly selected *M. pyrifera* individuals from each site had approximately 0.5 g of tissue removed from their apical meristem region (n = 5). This piece of tissue then had its wet weight taken prior to being wrapped in aluminum foil and freeze dried. Samples were freeze dried as preliminary experiments showed better pigment extraction with freeze dried tissue as opposed to fresh tissue. Following freeze drying, tissue samples were reweighed, placed in labeled glass test tubes (10% HCl washed) and ground into fine flakes using a small glass rod. Each test tube then had four ml of dimethyl sulfoxide (DMSO) and one ml of high purity water added to it before being covered with Parafilm®. The test tubes were then placed in a centrifuge at 3000 rpm for three minutes. The extract in each test tube was decanted into clean labeled test tubes and four ml of this extract was then placed in a 5 ml glass cuvette. The extract had its absorbance measured in a Pharmacia Biotech Ultrospec® 2000 (Pharmacia Biotech, Sweden) spectrophotometre at 665, 631, 582 and 480 nm using a blank of four parts DMSO and one part high purity water. Three ml of 90% acetone, one ml of methanol and one ml of high purity water was then added to the tissue in the test tubes. After a period of 30 minutes with periodic shaking, the extract was decanted off. The absorbance of the extract was measured in a 5 ml glass cuvette at 664, 631, 581 and 470 nm using a blank of three ml 90% acetone and one ml of methanol. The values obtained were then entered into a spreadsheet and pigment concentrations calculated per milligram of dry weight using formula outlined in Seely et al. (1972).

(3.2.9) Isotope mixing model

Isotope mixing models have been used to delineate how different sources of nitrogen are utilised by organisms (Phillips and Koch 2002; Philips et al. 2005). An IsoSource two source isotope mixing model was used to estimate the extent to which the salmon farm and mussel farm are contributing to the total amount of nitrogen sequestered by the experimentally grown *M. pyrifera* (Phillips and Kock 2003; Philips et al. 2005). The formula for the mixing model is: $\delta^{15}N_x = X (\delta^{15}N_{salmon\ pellet/mussel\ tissue}) + (1-X) \delta^{15}N_y$ where X is the fraction of nitrogen derived from the salmon pellets or mussel waste, $\delta^{15}N_x$ is the average $\delta^{15}N$ value of the *M. pyrifera* grown near the salmon or mussel farm (the mixture), $\delta^{15}N_{salmon\ pellet/mussel\ tissue}$ is the $\delta^{15}N$

signature of the salmon pellet/mussel waste (source 1), and $\delta^{15}N_y$ is the $\delta^{15}N$ value of the macroalgae grown at the control site (source 2).

(3.2.10) Data analysis

To determine whether there was a statistically significant difference in *M. pyrifera* growth rate, pigment concentrations, nitrogen and carbon status and isotopic signatures between sites and seasons, a two-way analysis of variance (ANOVA) was performed ($\alpha = 0.05$) (Zar 1996). Statistically significant differences among means were determined using a Tukey's post-hoc test (Zar 1996). A one-way ANOVA and Tukey post-hoc tests were used to test for statistically significant differences in water chemistry between growing sites (Zar 1996). Data were tested for normality (D'Agostino-Pearson omnibus test) before any statistical analysis took place and all data were normally distributed. Statistical analysis of data was performed using GraphPad Prism 6®. To determine the extent to which the experimentally grown *M. pyrifera* are using nitrogen originating from the selected salmon and mussel farm at Big Glory Bay, an IsoSource isotope mixing model was used (Phillips and Koch 2002; Philips et al. 2005) (see section 2.2.9).
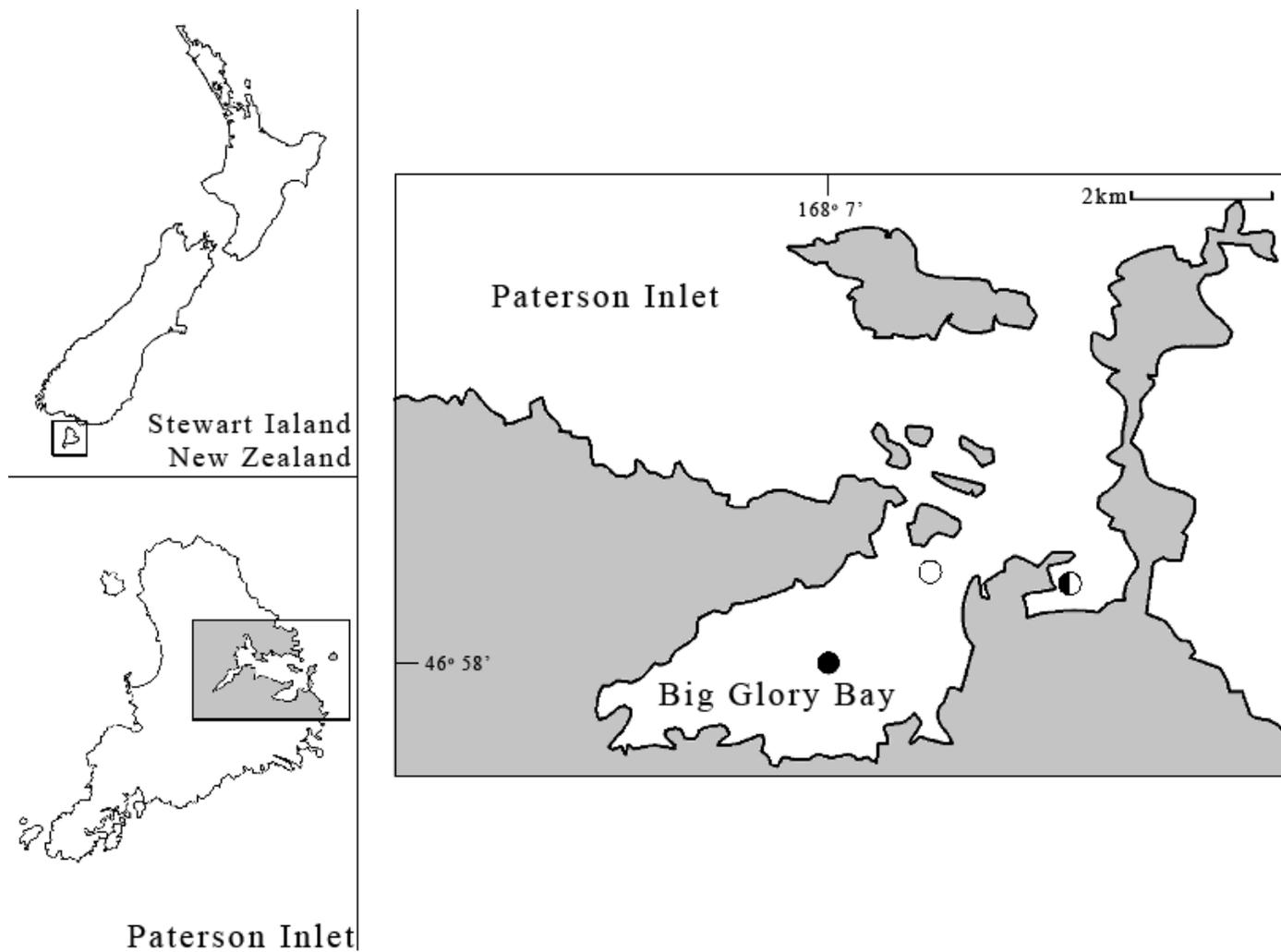
Figure 3.1: Map of New Zealand, Stewart Island and Paterson Inlet showing the location of the experimental growing sites within Paterson Inlet.

● salmon site, o mussel site, ◐ control site.

Figure 3.2: *M. pyrifera* growing frame used to conduct IMTA trials in Big Glory Bay, Stewart Island, New Zealand

(3.3) Results

(3.3.1) Seawater chemistry and light data

Ammonium concentrations at the salmon, mussel and control sites did not differ significantly in both summer and autumn (Table 3.1). However, summer seawater ammonium concentrations at the salmon farm site showed large amounts of variability with measurements ranging from 0.6 μM to 12.8 μM whereas summer ammonium concentrations from the control site ranged from 0.6 μM to 2.4 μM. Nitrate concentrations did not differ significantly between sites during summer and early autumn (April) but in May, nitrate concentrations were highest at the salmon site and there was a statistically significant difference in nitrate concentrations found between the salmon and mussel site, the salmon and control site and the mussel and control site (Table 3.1). Phosphate concentrations were low at all sites (<1.1 μM) and were significantly higher at the control site during summer but did not differ significantly between sites in both April and May (Table 3.1).

Readings from the light loggers produced erroneous data and it was deduced that this was due to the light loggers not sitting correctly (level) on the growing frames.

(3.3.2) Growth rate

There was no significant effect of growing site or season on *M. pyrifera* RGR (Figure 3.3, Table 3.2). In the summer, *M. pyrifera* RGR was higher at the salmon site with the average RGR at the salmon site just over three times greater than the *M. pyrifera* RGR at the control site but this was not significant (Figure 3.3). Autumn *M. pyrifera* growth rates at each site were similar (Figure 3.3).

(3.3.3) Seasonal carbon and nitrogen status

M. pyrifera percentage nitrogen and C:N differed significantly by growing site and season while *M. pyrifera* percentage carbon did not differ significantly by growing site or season (Figure 3.4a,b,c, Table 3.2). During the summer growing period, average total *M. pyrifera* tissue nitrogen content was 1.6 times higher at the salmon site compared to the control site,

while total tissue nitrogen content at the control and mussel sites was comparable (Figure 3.4a). In autumn, *M. pyrifera* percentage nitrogen increased significantly at the mussel site while *M. pyrifera* percentage nitrogen at the salmon and control did not differ significantly between seasons (Figure 3.4a).

There was an effect of growing site and season on *M. pyrifera* C:N (Figure 3.4c). In summer, the average C:N ratio of the *M. pyrifera* grown at the mussel and control site was 1.7 and 1.9 times higher respectively than the average C:N of the *M. pyrifera* grown at the salmon farm site (Figure 3.4c). In autumn, the *M. pyrifera* C:N decreased at the mussel site while the C:N at the control and salmon sites were similar during summer and autumn (Figure 3.4c).

(3.3.4) $\delta^{15}N$ analysis

During the summer growing period, the $\delta^{15}N$ signature of *M. pyrifera* from the salmon farm site was not significantly different from $\delta^{15}N$ signature of the *M. pyrifera* grown at the mussel and control sites (Figure 3.5). However, the summer $\delta^{15}N$ signature of the *M. pyrifera* grown at the salmon site was closer to the $\delta^{15}N$ signature of the salmon pellets (Figure 3.5). In autumn the *M. pyrifera* $\delta^{15}N$ signature increased consistently across all sites (interaction effect was not significant (Table 3.2)) and the seasonal increase at each site was not significant (Figure 3.5). The autumn $\delta^{15}N$ signature of *M. pyrifera* from the salmon site diverged away from the salmon pellet $\delta^{15}N$ signature (Figure 3.5).

(3.3.5) Soluble nitrogen

M. pyrifera soluble ammonium and nitrate differed seasonally and with growing site (Figure 3.6a, Table 3.3). During the summer, *M. pyrifera* from the salmon site had significantly higher soluble ammonium concentrations compared to *M. pyrifera* from the mussel and control sites and *M. pyrifera* from the control site had significantly higher soluble ammonium concentrations compared to the mussel site (Figure 3.6a). In autumn however, soluble ammonium concentrations were comparable amongst sites and were lower in autumn compared to summer at the salmon and control sites (Figure 3.6a). In summer, *M. pyrifera* soluble nitrate concentrations were comparable amongst sites (Figure 3.6b). In autumn, soluble nitrate

concentrations were significantly higher at the salmon site compared to the control site (Figure 3.6b).

(3.3.6) Pigments

(3.3.6.1) Chlorophyll *a*

M. pyrifera chlorophyll *a* concentrations varied significantly by season and growing site (Figure 3.7a, Table 3.3). In summer there was no significant difference in the *M. pyrifera* chlorophyll *a* concentrations amongst sites (Figure 3.7a). However, during autumn *M. pyrifera* chlorophyll *a* concentrations were significantly elevated at the salmon site relative to the control site (Figure 3.7a). At all growing sites, chlorophyll *a* concentrations were significantly higher during the autumn compared to the summer.

(3.3.6.2) Chlorophyll *c*

M. pyrifera chlorophyll *c* concentrations were not significantly affected by growing site (Figure 3.7b, Table 3.3). However, at all growing sites chlorophyll *c* concentrations were significantly higher during the autumn compared to the summer (Figure 3.7b, Table 3.3).

(3.3.6.3) Fucoxanthin

M. pyrifera fucoxanthin concentrations varied significantly by growing site and season (Figure 3.7c, Table 3.3). In summer there was no significant difference in the *M. pyrifera* fucoxanthin concentrations amongst sites (Figure 3.7c). However, during autumn *M. pyrifera* fucoxanthin concentrations were elevated at the salmon site relative to the control site (Figure 3.7c). At the salmon and mussel site, fucoxanthin concentrations were significantly higher during the autumn compared to the summer but significant seasonal differences in fucoxanthin concentrations did not occur for *M. pyrifera* from the control site (Figure 3.7c).

(3.3.7) δN^{15} mixing model

Analysis of salmon farm *M. pyrifera* tissue using a two source isotope mixing model revealed that during summer, on average 68% of nitrogen sequestered by *M. pyrifera* grown at the salmon site was of salmon farm origin (from the salmon pellets) (Table 3.4). In autumn the average proportion of salmon farm-derived nitrogen sequestered by the *M. pyrifera* from the salmon site reduced to 26% (Table 3.4). Analysis of *M. pyrifera* tissue from the mussel site using the two source isotope mixing model produced erroneous data which suggests more than two sources of nitrogen are contributing to the total nitrogen sequestered by *M. pyrifera* grown at the mussel site.
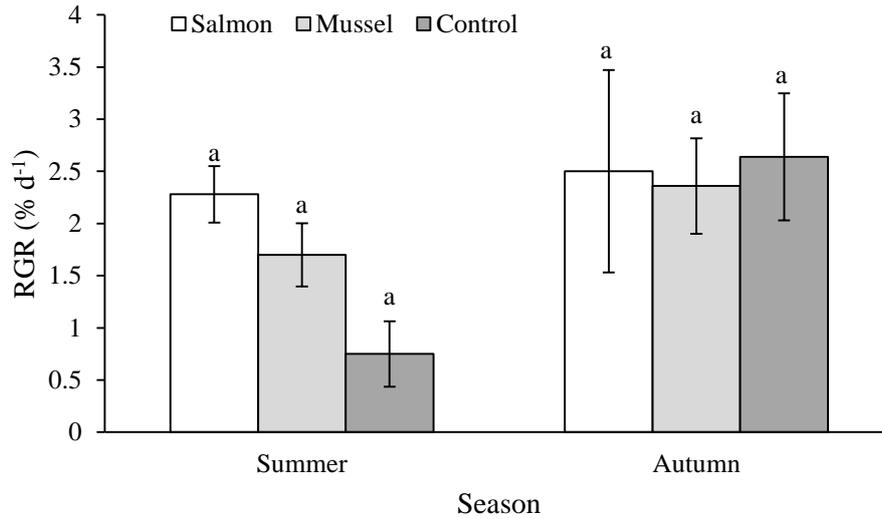
Figure 3.3: The average seasonal (summer and autumn) relative growth rate (RGR) of juvenile *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site at Big Glory Bay or Glory Cove, Stewart Island, New Zealand. Error bars ±1 S.E, n = 3 in the summer growing period, n = 4 in the autumn growing period. Different letters denote statistically significant differences between sites and seasons α = 0.05 (Tukey's post-hoc test).

Figure 3.4: The average seasonal (summer and autumn) percentage nitrogen (a), percentage carbon (b) and carbon to nitrogen ratio (C:N) (c) of juvenile *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site at Big Glory Bay or Glory Cove, Stewart Island, New Zealand. Error bars show ±1 S.E, n = 5, different letters denote statistically significant differences between sites and seasons α = 0.05 (Tukey's post-hoc test).

Figure 3.5: The average seasonal (summer and autumn) $\delta^{15}N$ content of salmon pellets and juvenile *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site at Big Glory Bay or Glory Cove, Stewart Island, New Zealand. Error bars ± 1 S.E, n = 5, different letters denote statistically significant differences ($\alpha = 0.05$, Tukey's post-hoc test).
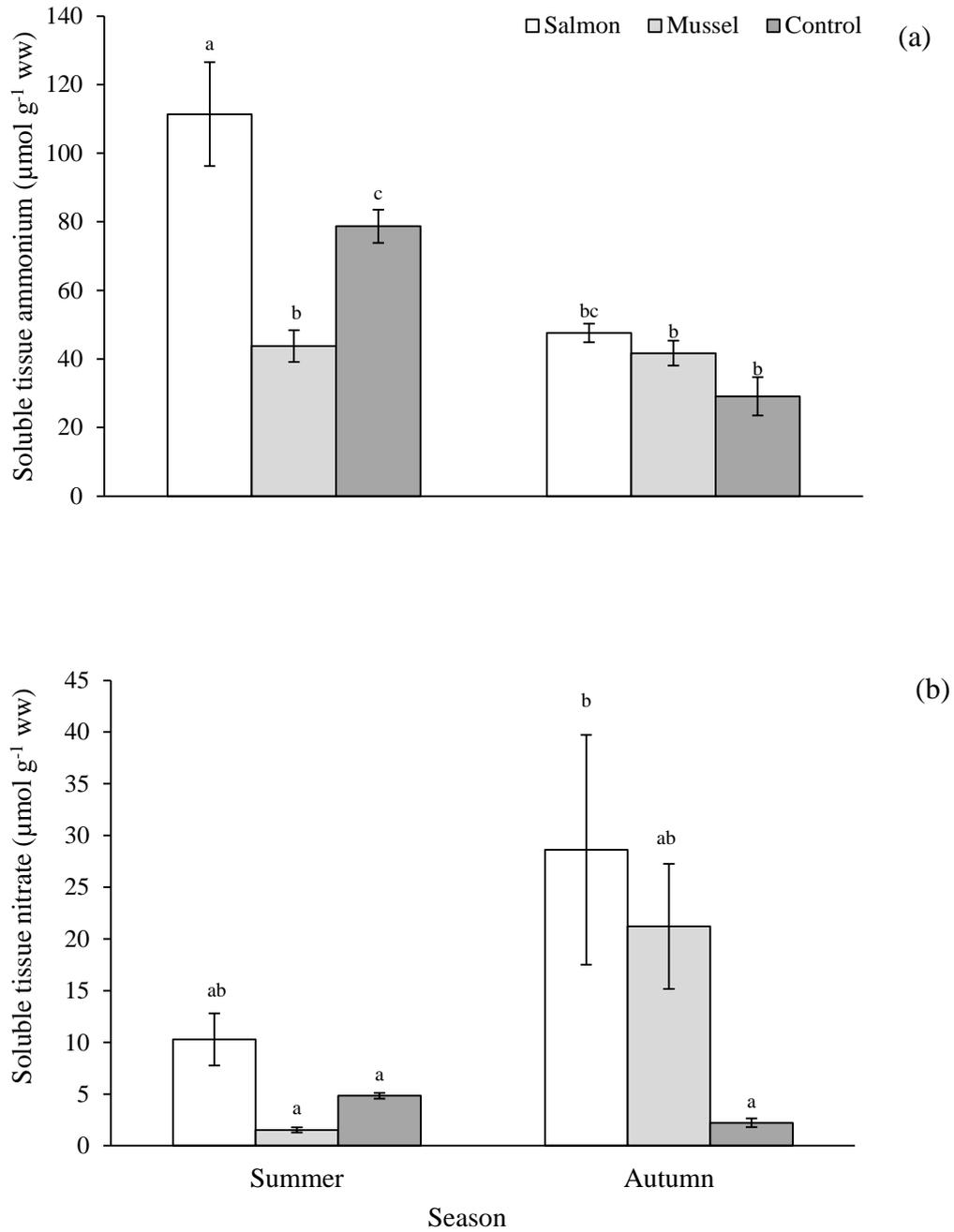
Figure 3.6: The average seasonal soluble ammonium (a) and nitrate (b) concentrations of juvenile *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site at Big Glory Bay or Glory Cove, Stewart Island, New Zealand. Error bars show ±1 S.E, n = 5, different letters denote statistically significant differences between sites and seasons (α = 0.05 Tukey's post-hoc test).

Figure 3.7: Average seasonal (summer, autumn) chlorophyll *a* (a), chlorophyll *c* (b) and fucoxanthin (c) concentrations of *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site at Big Glory Bay or Glory Cove, Stewart Island, New Zealand. Error bars show ±1 S.E, n = 5, different letters denote statistically significant differences between sites and seasons ($\alpha = 0.05$ Tukey's post-hoc test).

Table 3.1: Water chemistry results for around a salmon farm and mussel line in Big Glory Bay and a control site at Glory Cove, Stewart Island, New Zealand.

	Summer (December)			Autumn (April)			Autumn (May)		
	NH_4^+	NO_3^-	PO_4^{3-}	NH_4^+	NO_3^-	PO_4^{3-}	NH_4^+	NO_3^-	PO_4^{3-}
Salmon	3.10±0.91	0.76±0.11	0.35±0.03	1.89±0.18	0.91±0.06	0.35±0.03	1.53±0.04	2.81±0.23	0.83±0.02
Mussel				2.19±0.37	0.91±0.07	1.10 ±0.10	2.48±0.44	1.98±0.16	0.83±0.06
Control	1.30 ±0.24	0.59±0.10	0.74±0.03	1.33±0.32	0.94±0.06	0.35±0.03	1.98±0.29	0.68±0.09	0.79±0.08
Within season significance			*S-C					*S-M *M-C *S-C	

Water chemistry (NH_4^+, NO_3^-, PO_4^{3-} μM) results for the salmon, mussel and control sites during different seasons. Values are averages ± 1 S.E, n = 5-14. 'Within season significance' outlines whether there was a statistically significant difference between sites, within a season, for a nutrient type. An asterisk (*) indicates a significance difference (α = 0.05 Tukey's post-hoc test) and the initials S (salmon), M (mussel) and C (control) indicate the sites in which the statistically significant difference applies. Summer water samples from the salmon farm site were taken from directly next to the salmon farm and April and May water samples were taken at the seaweed growing site located ~25 metres away from the salmon farm edge. Due to different sampling locations each season, seasonal differences in nutrient concentrations could not be explored statistically.

Table 3.2: Two-way ANOVA for the seasonal (summer and autumn) growth (RGR), percentage nitrogen and carbon, C:N and $\delta^{15}N$ of *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site in Big Glory Bay or Glory Cove, Stewart Island, New Zealand.

Factor	*F*	*df*	p
Growth (RGR)			
Growing site	0.62	2, 14	0.55
Season	3.32	1, 14	0.09
Growing site × Season	0.97	2, 14	0.40
Percentage Nitrogen			
Growing site	35.48	2, 24	<0.001
Season	25.17	1, 24	<0.001
Growing site × Season	18.35	2, 24	<0.001
Percentage Carbon			
Growing site	0.59	2, 24	0.568
Season	0.84	1, 24	0.368
Growing site × Season	1.51	2, 24	0.242
C:N			
Growing site	28.87	2, 24	<0.001
Season	8.95	1, 24	0.006
Growing site × Season	16.29	2, 24	<0.001
$\delta^{15}N$			
Growing site	6.79	2, 24	0.005
Season	11.87	1, 24	0.002
Growing site × Season	0.63	2, 24	0.539

Table 3.3: Two-way ANOVA for the seasonal (summer and autumn) soluble nitrogen (ammonium, nitrate) and pigments (chlorophyll *a*, chlorophyll *c*, fucoxanthin) of *M. pyrifera* grown at three different sites: a salmon farm site, a mussel farm site and a control site in Big Glory Bay or Glory Cove, Stewart Island, New Zealand.

Factor	*F*	*df*	p
Soluble Ammonium			
Growing site	12.18	2, 23	<0.001
Season	39.33	1, 23	<0.001
Growing site × Season	9.30	2, 23	0.001
Soluble Nitrate			
Growing site	5.85	2, 23	0.009
Season	9.82	1, 23	0.005
Growing site × Season	3.76	2, 23	0.039
Chlorophyll *a*			
Growing site	5.80	2, 24	0.008
Season	79.78	1, 24	<0.001
Growing site × Season	3.48	2, 24	0.048
Chlorophyll *c*			
Growing site	0.325	2, 24	0. 726
Season	162.1	1, 24	<0.001
Growing site × Season	0.258	2, 24	0.775
Fucoxanthin			
Growing site	3.67	2, 24	0.041
Season	56.55	1, 24	<0.001
Growing site × Season	4.38	2, 24	0.024

Table 3.4: Results from a two source isotope mixing model showing the seasonal contribution of salmon farm (salmon pellets) and naturally derived nitrogen to *M. pyrifera* grown outside a salmon farm in Big Glory Bay, Stewart Island, New Zealand.

	Summer	Autumn
Proportion of salmon farm-derived nitrogen	0.680±0.132	0.259±0.228
95% confidence interval for proportion of salmon farm-derived nitrogen	[0.370, 0.991]	[0, 0.799]
Proportion of naturally derived nitrogen	0.320±0.132	0.740±0.228
95% confidence interval for proportion of naturally derived nitrogen	[0.009, 0.632]	[0.201, 1]

Values are averages ± 1 S.E, n = 5.

(3.4) Discussion

Results suggest that the Kiwa 1 salmon farm can provide nitrogen for use by the co-cultured *M. pyrifera* particularly during the summer low seawater nitrogen period. In summer, evidence indicates that the salmon farm can provide additional nitrogen as the *M. pyrifera* tissue nitrogen content and soluble ammonium concentrations were higher at the salmon site compared to the mussel and control sites. There was some evidence to suggest that the additional nitrogen could be supporting *M. pyrifera* growth during the summer low seawater nitrogen period but results were statistically inconclusive. Analysis of $\delta^{15}N$ signatures indicates that on average, 68% of nitrogen sequestered by *M. pyrifera* from the salmon site during summer is of salmon farm origin. In autumn however, naturally occurring seawater nitrogen concentrations are increasing and this appeared to negate the effect of salmon farm nitrogen provision on *M. pyrifera* growth, nitrogen status and sequestration of salmon farm-derived nitrogen. It is also possible that reduced light during autumn is limiting growth and hence additional nitrogen will have a limited effect on *M. pyrifera* growth.

(3.4.1) Seasonal seawater nitrogen and phosphorous patterns

During both summer and autumn, seawater samples from the salmon site did not show significantly elevated seawater ammonium concentrations relative to the control site. However, there was some trending towards higher seawater ammonium concentrations at the salmon site during summer. The highest concentration of ammonium found in a salmon farm water sample was 12.8 μM whereas the highest concentration of ammonium found in a control site water sample was 2.4 μM. In this study, the range of ammonium concentrations found in the salmon farm water samples (0.6-12.8 μM) is consistent with other studies that have documented the ammonium concentrations in water samples taken from outside salmon farms; Wildish et al. (1993) 2-10 μM, Petrell and Alie (1996) 2-7.5 μM, Ahn et al. (1998) 1-34 μM, Sanderson et al. (2008) 2-8 μM, Wang et al. (2014a) 0-6.8 μM. Typically, elevated ammonium concentrations are observed outside finfish farms (relative to control sites) and this is attributed to fish metabolic activity and leaching from fish feed (Wu et al. 1994; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Sanderson et al. 2008; Handå et al. 2013; Wang et al. 2014a). Possible explanations for lack of significantly elevated seawater ammonium

concentrations occurring at the salmon site include the sampling protocol used, the timing of sampling events and site characteristics.

To obtain a measure of ammonium in the water column, water samples were taken at the water surface only. It is possible that the ammonium concentration in the water column could change at different depths. Some comparable studies that have found elevated ammonium concentrations outside fish farms have taken water samples at different water column depths (Wu et al. 1994; Pitta et al. 2006; Mantzavrakos et al. 2007; Navarro et al. 2008; Sanderson et al. 2008) and is a potential reason for the differing results found in this study. There is also the possibility that the timing of the sample taking at the salmon farm site affected the observed ammonium concentrations. Research has suggested that peaks in farmed finfish ammonium production are correlated with feeding (Ahn et al. 1998; Pitta et al. 1999; Sanderson et al. 2008). The water samples in this study may not have been taken during or immediately after feeding which could have affected the measured seawater ammonium concentration. Furthermore, *in situ* water movement may also explain the lack of significantly elevated seawater ammonium concentrations at the salmon site. A wind driven current speed of $0.02m^{-1}$ is present within Big Glory Bay (Russell 2013, unpublished data) and this could be sufficient to rapidly disperse the salmon farm-derived ammonium. Wu et al. (1994) and Pitta et al. (1999) found that sufficient water movement at some of their studied fish farms diluted the ammonium in the water to an extent to which ammonium in their fish farm seawater samples was not significantly different from their control sites.

Seawater nitrate concentration at the salmon site was comparable to the control site during summer and April (early autumn). During the May (autumn) sampling period, there was evidence of increased seawater nitrate concentrations at the salmon and mussel site but not at the control site. Lack of increasing autumn seawater nitrate concentration at the control is not typical of Southern New Zealand and temperate regions generally. Usually there are low seawater nitrate concentrations during summer and increased or increasing seawater nitrate concentrations during autumn and/or winter (Wheeler and North 1981; Wheeler and Srivastava 1984; van Tussenbroek 1989; Brown et al. 1997; Phillips and Hurd 2003; Hepburn and Hurd 2005; Hepburn et al. 2007; Stephens and Hepburn 2014). It is important to note that the samples taken in this study are isolated sampling events and may not be representative of actual nitrate concentrations in the water column. To build a more accurate picture of ambient nitrate concentrations at the control site, more water samples would need to be taken over a longer

time period. However, it is noteworthy that during autumn there was evidence of low tissue nitrogen and soluble nitrate in the *M. pyrifera* from the control site (see sections 3.4.3.1 and 3.4.3.2) and this provides further evidence of low seawater nitrate concentrations at the control site during autumn. The cause of low autumn seawater nitrate concentrations at the control site is unknown but could be due to localised hydrodynamic conditions.

Seawater phosphate concentrations were low at all sites but observation of the seawater nitrogen:phosphorous (N:P) revealed that phosphorous was not limiting. For macroalgae generally, the optimal N:P ratio is 30:1 (but can range from 10:1 to 80:1) (Atkinson and Smith 1983). During each season and at each site the seawater N:P was <10:1. This ratio suggests that phosphorous was not limiting *M. pyrifera* growth (Harrison and Hurd 2001). Seawater phosphate concentrations were similar amongst sites during autumn however the control site seawater phosphate concentrations were higher than salmon farm seawater phosphate concentrations in summer. This contrasts with other comparable studies that have examined how phosphate concentrations change with the proximity of a finfish farm. Phosphate concentrations can be elevated outside finfish farms compared to control sites and this is attributed to fish metabolic activity (Wu et al. 1994; Chopin et al. 1999; Pitta et al. 1999; Pitta et al. 2006; Mantzavrakos et al. 2007). Possible explanations for lack of significantly elevated phosphate concentrations at the salmon site are the same as for ammonium (section 3.4.1), namely the sampling protocol used, the timing of sampling and site characteristics.

(3.4.2) Seasonal growth rate

(3.4.2.1) Seasonal growth at the salmon and control sites

M. pyrifera growth rate showed some variation seasonally and with growing site but the differences in growth rate amongst sites and seasons were not significantly different. Lack of a significantly higher *M. pyrifera* growth rate at the salmon site is not consistent with comparable studies that have experimentally grown macroalgae outside salmon farms and at control sites, then compared key parameters in the interest of IMTA. Faster growth rates for macroalgae co-cultured with salmon has been observed in Canada (*Saccharina latissima*) (Chopin et al. 2004), Scotland (*S. latissima*, *Palmaria palmata*) (Sanderson et al. 2012), Norway (*S. latissima*) (Handå et al. 2013; Wang et al. 2014a) and Chile (*Gracilaria chilensis*) (Troell et al. 1997; Abreu et al. 2009). Faster macroalgal growth rates outside finfish farms are

attributed to higher dissolved nitrogen concentrations in the waters surrounding the finfish farm (as a consequence of fish metabolic activity) and this additional nitrogen supplements macroalgal growth particularly when growth is nitrogen limited. Although there was no significant difference in the seawater ammonium concentration amongst sites, there was still some evidence of higher ammonium concentrations at the salmon site (see section 3.4.1) so lack of significantly elevated *M. pyrifera* growth at the salmon site is unusual.

Despite the lack of a significantly greater *M. pyrifera* growth rate at the salmon site, in summer there was some evidence of enhanced *M. pyrifera* growth at the salmon site compared to the control site. In summer, *M. pyrifera* growth was on average ~3.0 times greater at the salmon site compared to the control site. In southern New Zealand, *M. pyrifera* growth is typically nitrogen limited during the summer (Brown et al. 1997; Hepburn et al. 2007; Stephens and Hepburn 2014) and nitrogen limitation is an important parameter affecting both juvenile and adult *M. pyrifera* growth and survivorship (Wheeler and North 1980; Dean and Jacobsen 1984; Dean and Jacobsen 1986; Zimmerman and Kremer 1986; Brown et al. 1997; Hernández-Carmona et al. 2001; Hepburn et al. 2007; Stephens and Hepburn 2014). It is possible that additional nitrogen (in the form of ammonium) supplied by the salmon farm could explain why there was trending towards a greater summer *M. pyrifera* growth rate at the salmon site compared to the control site. Nitrogen supplementation from the salmon farm could have supported *M. pyrifera* growth (to an extent) at the salmon site during the summer period of nitrogen limitation.

During the autumn growing season there was no significant difference in *M. pyrifera* growth rate between sites. This was expected as natural seawater nitrogen levels would be increasing during this time and light would be decreasing. Nitrogen limitation would theatrically not be a problem during this time but rather growth could be light limited. However, at the control site low seawater nitrate concentrations remained during autumn. In southern New Zealand seawater nitrate concentrations are usually increasing or are increased during this time (Brown et al. 1997; Hepburn et al. 2007). Additionally, the percentage nitrogen and soluble nitrate concentrations in *M. pyrifera* from the control site remained low during autumn (discussed in sections 3.4.3.1 and 3.4.3.2) providing further evidence that the autumn seawater nitrate concentration was low at the control site. Sustained *M. pyrifera* growth at the control site despite low ambient nitrogen concentrations could be attributed to the initial nitrogen reserves of the *M. pyrifera*. Gerard (1982c) has documented sustained adult *M. pyrifera* growth over

short time periods (two weeks) despite low seawater nitrogen concentrations and the sustained growth was attributed to nitrogen reserves.

The unexpected growth results in this study, namely the lack of a significantly higher *M. pyrifera* growth rate at the salmon site and absence of a significantly higher *M. pyrifera* autumn growth rate across sites, could be explained by the small sample sizes used in this study. Only three replicate growing frames were used per site in summer and four per site in autumn. Additionally, in autumn the *M. pyrifera* from one growing frame at the salmon site was lost (presumably due to grazing by invertebrates). The small sample size used and the missing data may explain (at least in part) why a significantly increased *M. pyrifera* growth rate was not observed at the salmon farm site.

(3.4.2.2) Seasonal growth at the mussel and control sites

During both the summer and autumn growing season, there was no significant difference in the growth rate of the *M. pyrifera* grown at the mussel and control site. Lack of significantly enhanced *M. pyrifera* growth at the mussel site differs with the findings of other studies that have analysed how macroalgal growth rate responds to nitrogen provision by bivalves. In a natural setting, the macroalga *Porphyra perforata* has a faster growth rate when grown on living mussel *Mytilus californianus* compared to when grown on *M. californianus* mimics and bare rock (Aquilino et al. 2009). This faster growth rate is attributed to nitrogen provision by *M. californianus*. In laboratory and mesocosm experiments, nitrogen provision by scallops (*Chlamys farreri*), mussels (*M. californianus*) and clams (*Tapes philippinarum*) has been found to enhance macroalgae *Gracilaria lemaneiformis* (Mao et al. 2009), *Odonthalia floccosa* (Bracken 2004) and *Ulva rigida* (Bartoli et al. 2003) growth. However, these macroalgae are much smaller than *M. pyrifera* and hence their nitrogen demand may be lower. Consequently, small amounts of nitrogen provision from bivalves may be sufficient enough to enhance smaller macroalgal species growth but possibly not the growth of larger macroalgal species with potentially higher nitrogen demands, namely *M. pyrifera*.

The lack of a significantly increased *M. pyrifera* summer growth rate at the mussel site compared to the control site could be due to the positioning of the growing frames. Specifically, the growing frames may not have been deployed close enough to active mussel lines. Because of mussel harvesting activities in the area, the *M. pyrifera* growing frames had to be deployed

on an inactive mussel line (close to active mussel lines but not directly next to them). It is possible that the nitrogen provided by the mussels in the area was diluted by water movement to an extent that the nitrogen provision was reduced. It is noteworthy that at the entrance to Big Glory Bay (the location of the mussel site) current speed is much greater (0.15 ms^{-1}) than further in the bay (Russell 2013, unpublished data). As with the salmon farm, during autumn it is likely that higher ambient nitrogen concentrations negated any effect of mussel nitrogen provision on *M. pyrifera* growth and the presumably reducing light levels limited growth.

These results suggest that large kelp species (such as *M. pyrifera*) may not be suited to open water IMTA with bivalves only, particularly in Big Glory Bay. Because of water movements dispersing the mussel-derived nitrogen and the large size *M. pyrifera* can achieve, *M. pyrifera* may not be able to be cultivated close enough to the mussels to enable effective sequestration of their nitrogenous waste and hence gain enhanced growth.

(3.4.3) Seasonal *M. pyrifera* nitrogen status

(3.4.3.1) *M. pyrifera* percentage nitrogen and carbon to nitrogen ratio

The presence of the salmon farm affected the co-cultured *M. pyrifera* nitrogen status. This was demonstrated by higher percentage nitrogen content, higher soluble ammonium concentrations and lower C:N for *M. pyrifera* from the salmon site relative to the control site. This effect was especially pronounced during the summer growing period. Typically, macroalgae that are grown near or collected from finfish farms have higher nitrogen content in their tissues and lower C:N compared to macroalgae grown at or collected from control sites (Troell et al. 1997; Chopin et al. 1999; Abreu et al. 2009; García-Sanz et al. 2010; García-Sanz et al. 2011; Sanderson et al. 2012). The higher percentage nitrogen content and lower C:N can be attributed to the additional nitrogen supplied by the fish metabolic activity and nitrogen leaching from salmon feed which supplies nitrogen for assimilation by the co-cultured *M. pyrifera*.

Despite provision of nitrogen by the salmon farm during summer, the C:N of the *M. pyrifera* from the salmon site suggests nitrogen limitation may still be a factor affecting growth. For macroalgae, a C:N ratio of fifteen or more is thought to indicate nitrogen limitation (Hanisak 1983). Observation of the average summer C:N ratios indicates that there is evidence of nitrogen limitation at all three sites (salmon C:N = 17, mussel C:N = 32, control C:N = 28).

However, Lapointe & Duke (1984) have argued that such a nitrogen limitation threshold should not be applied to macroalgae as nitrogen required for growth is affected by light and notably, Stewart Island daylight hours are high particularly during summer and waters are clear (Desmond et al. in press). It thus appears that either growth is nitrogen limited at all sites or Stewart Island *M. pyrifera* nitrogen requirements are lower than Hanisak's (1983) threshold because of high Stewart Island light levels. Regardless, the significantly lower *M. pyrifera* C:N ratio observed at the salmon site and comparable *M. pyrifera* tissue carbon content across sites, provides evidence that *M. pyrifera* is utilising nitrogen derived from the salmon farm during summer when naturally occurring seawater nitrogen concentration is at its lowest.

In autumn the percentage nitrogen content of *M. pyrifera* from the salmon and mussel sites increased and at the mussel site there was a significant difference in the seasonal percentage nitrogen content and C:N. A lower percentage nitrogen content in summer and increased percentage nitrogen content in autumn/winter is typical of *M. pyrifera* (Wheeler and Srivastava 1984; van Tussenbroek 1989; Brown et al. 1997; Hepburn et al. 2007; Stephens and Hepburn 2014). It can be deduced that increased autumn *M. pyrifera* percentage nitrogen content at these sites is a result of higher seawater nitrate concentrations that were evident during this time. The control site however did not show the same pattern of increased *M. pyrifera* percentage nitrogen content during the autumn and there was evidence of low autumn seawater nitrate concentration at the control site (see section 3.4.1). This is not typical of Stewart Island and Southern New Zealand (Brown et al. 1997; Phillips and Hurd 2003; Hepburn et al. 2007; Stephens and Hupburn 2014) and some localised hydrodynamic conditions could be affecting typical seawater chemistry patterns which in turn are affecting the percentage nitrogen content of *M. pyrifera* from the control site.

The presence of the mussel farm had no significant effect on *M. pyrifera* nitrogen status. In summer, the average percentage nitrogen and C:N of *M. pyrifera* from the mussel site did not differ significantly from the control site and suggests that the mussels are not contributing a significant amount of nitrogen to the *M. pyrifera* grown at this site. This contrasts with studies that have documented how macroalgal percentage nitrogen content changes with nitrogen provision from bivalves. Laboratory (Bartoli et al. 2003; Bracken 2004; Mao et al. 2009) and field experiments (Aquilino et al. 2009) have shown that macroalgae grown alongside bivalves can have elevated percentage nitrogen content in their tissues and this is attributed to nitrogen provision from bivalve excretion. A possible reason why no elevated percentage nitrogen

content was observed in the *M. pyrifera* from the mussel site is the proximity of the *M. pyrifera* growing frames to active mussel lines. As mentioned in section 3.4.2.2, the growing frames were deployed on experimental mussel lines that did not have mussels growing on them at the time (but the growing frames were still relatively close to active mussel lines). It is likely that any provision of nitrogen from the nearby mussels was diluted to an extent to which the mussels did not provide sufficient nitrogen to elevate the percentage nitrogen content of the *M. pyrifera* grown at the mussel site.

(3.4.3.2) Soluble tissue ammonium and nitrate

Higher soluble ammonium concentrations in summer relative to summer soluble nitrate concentrations have been observed for *M. pyrifera* from southern New Zealand and this is attributed to the ammonium which remains in the water column during the summer period and pulses of ammonium which become available, possibly from epifauna (Hepburn 2003). In autumn, the contribution of soluble ammonium to total soluble nitrogen reduced, particularly at the salmon and mussel sites (63% and 66% respectively) and this is likely due to the increased seawater nitrate concentrations occurring during this time. However, soluble ammonium still made up a great majority of total soluble nitrogen at the control site during autumn and it is likely that this is due to the absence of increased seawater nitrate concentrations that were evident at the control site (see section 3.4.1).

During summer, *M. pyrifera* soluble ammonium concentrations were significantly higher at the salmon site compared to the mussel and control sites. Provision of ammonium to macroalgae (whether naturally or experimentally provided) can result in elevated soluble ammonium concentrations in macroalgal tissue (Naldi and Wheeler 1999; Liu and Dong 2001; Hepburn 2003; Phillips and Hurd 2003). Higher summer *M. pyrifera* soluble ammonium concentrations at the salmon site suggests that ammonium from the salmon farm is being assimilated and stored intracellularly by the co-cultured *M. pyrifera* during this period when seawater nitrogen concentration is typically at its lowest. *M. pyrifera* grown at the control site had significantly higher soluble ammonium concentrations compared to *M. pyrifera* grown at the mussel site. This suggests that ammonium from mussel excretion was not a significant source of nitrogen for *M. pyrifera* grown at the mussel site or was not available in sufficient concentrations for significant *M. pyrifera* assimilation (perhaps due to dilution).

M. pyrifera pools of soluble nitrate followed seasonal patterns in seawater nitrate availability in most cases. In summer, soluble nitrate made up only 4-8% of total soluble nitrogen and reflects the limited availability of seawater nitrate during this time. In autumn, the seawater nitrate concentration increased at the salmon and mussel sites (relative to summer seawater nitrate concentration) and this was reflected in the size of the *M. pyrifera* soluble nitrate pools. Soluble nitrate made up 34% and 38% of total soluble nitrogen at the salmon and mussel site respectively. Increased soluble nitrate concentration in *M. pyrifera* from the salmon site is unusual given that theoretically, ammonium is energetically less expensive to take up relative to nitrate (Harrison and Hurd 2001) so it could be expected that at the salmon site *M. pyrifera* soluble ammonium concentrations would remain high. However, increased *M. pyrifera* soluble nitrate and reduced *M. pyrifera* soluble ammonium at the salmon site during the autumn could be due to naturally occurring nitrate being more consistently available compared to fish farm ammonium as ammonium production by fish farms can be variable and exhibit only peaks associated with feeding (Kelly et al. 1994; Ahn et al. 1998; Pitta et al. 1999; Sanderson et al. 2008). Despite this, patterns of low summer and higher autumn/winter soluble nitrate concentrations in kelp is typical and is attributed to the seasonal availability of nitrate (Chapman and Craigie 1977; Wheeler and Sirvastava 1984; Hepburn 2003; Young et al. 2007).

(3.4.4) Pigments

The absence of increased summer pigment concentrations at the salmon site was unexpected as nitrogen provision can result in increased macroalgal pigment content because pigments can be important nitrogen storage pools (Bird et al. 1982; Rosenberg and Ramus 1982; Shivji 1985; Stengel and Dring 1998; Harrison and Hurd 2001; Kim et al. 2007; Ribeiro et al. 2013). For *M. pyrifera*, laboratory studies have revealed that when both light and nutrient concentrations are high (conditions similar to what would be expected at the salmon site during summer), *M. pyrifera* pigment content can be elevated due to nitrogen storage via pigments (Shivji 1985). Absence of increased summer pigment concentrations at the salmon site suggests that in this instance pigments are not a significant nitrogen storage pool for *M. pyrifera* or alternatively, salmon farm-derived nitrogen was not being supplied in sufficient quantities to enable storage as pigments.

Autumn pigment concentrations increased significantly at each site except for fucoxanthin at the control site. Increased autumn *M. pyrifera* pigment content can be attributed to reduced light levels during this period as increased macroalgal pigment content in response to reduced light levels has been well documented (Ramus et al. 1976; Shivji 1985; Henley and Ramus 1989; Stengel and Dring 1998; Campbell et al. 1999; Stephens and Hepburn 2014). In autumn, chlorophyll *c* concentrations did not differ significantly between sites but there were significantly higher chlorophyll *a* and fucoxanthin concentrations for *M. pyrifera* grown at the salmon site compared to the control site. The increased *M. pyrifera* pigment content at the salmon site is unlikely to be due to increased nitrogen storage as pigments or summer *M. pyrifera* pigment content at the salmon site would have been higher as well. A more likely explanation for increased pigment content at the salmon site is the shading of the growing frames. The growing frames at the salmon site had to be deployed on some mooring lines that would have received late afternoon shading due to farm infrastructure. This shading and resulting reduced light levels could have prompted additional pigment production from the *M. pyrifera* grown at the salmon site as daylight hours would be reducing during this time.

(3.4.5) Salmon farm *M. pyrifera* $\delta^{15}N$ status and $\delta^{15}N$ mixing model

Normally those macroalgae that are grown outside finfish farms have heavier $\delta^{15}N$ signatures compared to control sites and this is attributed to the feed inputs and fractionation after fish feed consumption providing a heavier $\delta^{15}N$ signature (Vizzini and Mazzola 2004; García-Sanz et al. 2010; García-Sanz et al. 2011; Wang et al. 2014a). However, during the summer the *M. pyrifera* $\delta^{15}N$ signature from the salmon site was similar to the $\delta^{15}N$ signature of the salmon pellets. This is typical of macroalgal $\delta^{15}N$ signatures when they are cultured outside finfish farms; the $\delta^{15}N$ signatures move in the direction of the fish pellets and/or fish excretory products (Vizzini and Mazzola 2004; García-Sanz et al. 2010; Wang et al. 2014a). In contrast, the $\delta^{15}N$ signature of the *M. pyrifera* from the mussel and control sites differed from the salmon pellet $\delta^{15}N$ signature. These results suggest that during summer different nitrogen sources are being used by the *M. pyrifera* from the different sites. It is likely that *M. pyrifera* from the salmon farm site are taking up salmon farm-derived nitrogen whereas *M. pyrifera* from the mussel and control sites are taking up nitrogen from other sources, possibly only naturally occurring nitrogen.

The autumn *M. pyrifera* δ^{15}N signature was heavier at all sites compared to the summer *M. pyrifera* δ^{15}N signature and at the salmon site the *M. pyrifera* δ^{15}N signature diverged away from the δ^{15}N signature of the pellets. This result suggests that during the autumn, less salmon farm-derived nitrogen is being sequestered by the co-cultured macroalgae and they are taking up more naturally derived nitrogen during this time period when naturally occurring nitrogen concentrations are increasing.

During summer, the two source isotope mixing model indicated that on average 68% of the nitrogen sequestered by *M. pyrifera* was from the salmon pellets. However, during autumn the δ^{15}N signature of the *M. pyrifera* from the salmon farm diverged away from the salmon pellets δ^{15}N signature and the isotope mixing model indicated that the proportion of salmon farm-derived nitrogen sequestered by the *M. pyrifera* decreased to 26% during this time. This dilution is likely due to increased naturally occurring seawater nitrate and the possibility that natural nitrate is a more readily available nitrogen source as typically nitrogen (ammonium) from the salmon farm will be provided in intermittent pulses associated with feeding (Ahn et al. 1998; Pitta et al. 1999; Sanderson et al. 2008). Ultimately, the results from this isotope mixing model demonstrate that *M. pyrifera* can take up salmon farm-derived nitrogen but seasonal differences in nitrogen demand by *M. pyrifera* mean that nitrogen sequestration by *M. pyrifera* will be greatest during the summer period.

(3.5) Conclusions and recommendations

Results from this study suggest that *M. pyrifera* could be successfully used in an IMTA approach with salmon in Big Glory Bay, Stewart Island, New Zealand. Salmon farm-derived nitrogen can be sequestered by *M. pyrifera* and there is some evidence that this nitrogen can be used to support growth during seasonal periods of low ambient nitrogen. The co-culture of *M. pyrifera* with mussels in Big Glory Bay does not appear to be a viable IMTA option as there was limited evidence of mussel farm-derived nitrogen sequestration by *M. pyrifera*. Limited IMTA research has been performed in New Zealand and consequently future research should explore the possibility of co-culturing different species of macroalgae with salmon to try and determine the most appropriate IMTA species for both nutrient removal and economic gain. Larger scale experiments over a longer time period are warranted to further determine the potential for the IMTA of salmon and *M. pyrifera* in Big Glory Bay and to assess the economic viability of such an approach.

Chapter 4 *Macrocystis pyrifera* and its capacity for salmon farm-derived nitrogen sequestration

(4.1) Modeling *Macrocystis pyrifera* nitrogen uptake in Big Glory Bay

This study has generated estimates of *Macrocystis pyrifera* ammonium and nitrate uptake rate and confirmed that *M. pyrifera* is able to sequester salmon farm-derived nitrogen particularly during summer, when seawater nitrogen is at its lowest. The questions now remain: (1) how long will it take for *M. pyrifera* to reach maximum tissue nitrogen content given the salmon farm nitrogen input rate and *M. pyrifera* ammonium uptake rate? (2) how much *M. pyrifera* would need to be cultivated in summer and autumn to make a significant contribution to reducing nitrogen waste from the Kiwa 1 salmon farm, Big Glory Bay? This chapter combines data on the ammonium uptake rates of *M. pyrifera* (chapter 2) with seawater chemistry and *M. pyrifera* nitrogen status data from the IMTA trials (chapter 3) to address and answer these questions. This will provide insights into the short-term nitrogen enrichment buffering capacity of *M. pyrifera* and will outline the standing crop of *M. pyrifera* required to make a significant contribution to reducing nitrogen waste from the Kiwa 1 salmon farm. Additionally, addressing these questions will give further insight into the feasibility of an IMTA approach in Big Glory Bay, specifically, whether *M. pyrifera* is capable of taking up a significant amount of salmon farm-derived nitrogen, the extent to which the capacity for *M. pyrifera* nitrogen sequestration changes seasonally, and whether there would be sufficient *M. pyrifera* cultivation space in Big Glory Bay.

(4.2) How long will it take for *M. pyrifera* to reach maximum tissue nitrogen content?

M. pyrifera is unable to continually take up nitrogen despite a continuous supply and laboratory experiments have revealed that the maximum percentage nitrogen content attainable by *M. pyrifera* under nitrogen saturable conditions is approximately three percent (Wheeler and North 1980). Natural Stewart Island *M. pyrifera* populations can reach ~1.5-2.5% nitrogen (Hepburn et al. 2007; Stephens and Hepburn 2014). Estimating how long it would take for a standing crop of *M. pyrifera* to reach a maximum three percent nitrogen content given the Kiwa 1 salmon farm nitrogen input rate and *M. pyrifera* ammonium uptake rate, would demonstrate the short-term buffering capacity of *M. pyrifera* with respect to salmon farm nitrogen inputs.

(4.2.1) Estimate of Kiwa 1 salmon farm nitrogen input rate

When considering the input rate of nitrogen into Big Glory Bay from the Kiwa 1 salmon farm, sources of nitrogen include fish food (pellets), the fish themselves (their excretion) and the leaching of nitrogen from the aquaculture sediments (Figure 4.1). Estimates of nitrogen input into Big Glory Bay from these sources are calculated from data gathered in this study as well as other research that has been undertaken in Big Glory Bay.

Nitrogen inputs from the fish pellets. Salmon from the Kiwa 1 salmon farm are fed 14,000 kg of pellets per day (P. Nicholson, personal communication, February 2, 2015). On average that is 583.33 kg of pellets from the Kiwa 1 salmon farm entering Big Glory Bay per hour. In this study the pellets were analysed for percentage nitrogen content and are on average 6.6% nitrogen. 6.6% as a percentage of 583.33 kg is 38.50 kg which equates to a salmon pellet derived nitrogen input rate of 38.50 kg per hour. However, much of the nitrogen from the food inputs will be metabolised by the fish (Storebakken et al. 2000).

Nitrogen inputs from salmon excretion. In a study by Roper et al. (1988), a nutrient model for Big Glory Bay was produced. Roper et al. (1988), based on the findings of Weston (1986), estimate that 100 g of nitrogen is produced per kg of fish, per year. The Kiwa 1 salmon farm produces up to 2,400 tonne of salmon annually (P. Nicholson, personal communication, February 2, 2015) which results in 240,000 kg of nitrogen production per year as a result of fish excretion. That equates to a nitrogen production rate by the fish of 657.53 kg per day and 27.40 kg per hour.

Nitrogen inputs from the sediments under farm. Roper et al. (1988) determined an estimate of nitrogen leaching from Big Glory Bay salmon farm sediments. Nitrogen flux from aquaculture sediments under the Big Glory Bay farm is estimated to be 0.01 kg m^{-2} d^{-1}. The area under the Kiwa 1 salmon farm is approximately 10824 m^2. Hence, nitrogen released from this area is 108.24 kg per day which is 4.51 kg per hour.

Total nitrogen inputs. Total nitrogen input per hour = 27.40 kg (salmon nitrogen excretion) + 4.51 kg (sediment flux nitrogen). Total nitrogen input per hour = 31.91 kg h^{-1} (3.191^{10} µg h^{-1}).

For simplicity it is assumed that the 31.91 kg of salmon farm-derived nitrogen being released into Big Glory Bay each hour (some of which is in solid form) will be dissolved and distributed evenly throughout the water column under the salmon farm and that there will be no tidal flux. The volume of water under the farm is estimated to be 163,350 m^3 (surface area of farm 10890

m^2, depth 15 m) which equates to 163,350,000 L of water. Dissolving the nitrogen from the salmon farm into 163,350,000 L of water results in a seawater concentration and input rate of 195.35 µg L^{-1} h^{-1} (13.95 µmol L^{-1} h^{-1}).

Notably, there are several important factors that have the potential to affect the accuracy of the Kiwa 1 salmon farm nitrogen input rate estimate. Firstly, Roper et al. (1988) acknowledges that the uncertainty associated with their Big Glory Bay nutrient model could be as high as ± 50%. Secondly, this input rate calculation assumes that all solid nitrogen inputs are dissolved when realistically some of the nitrogen will remain in particulate form. Thirdly, it is likely that phytoplankton in the water column will take up nitrogen as it becomes available. Finally, salmon show diel variation in nitrogen excretion and peaks in nitrogen excretion associated with feeding (Kelly et al. 1994; Ahn et al. 1998; Pitta et al. 1999; Sanderson et al. 2008) and this has not been accounted for in the input rate calculations. Accounting for these factors is beyond the scope of this study but the nitrogen input estimate gives an indication of potential nitrogen input rates.

(4.2.2) Estimate of time required for *M. pyrifera* to reach three percent nitrogen

To estimate how long it would take for one kilogram (dry weight) of *M. pyrifera* to reach three percent tissue nitrogen content, the ammonium uptake rate at a substrate concentration of 13.95 µM (the input rate from the salmon farm) is required. Using the linear regression for ammonium uptake by *M. pyrifera* (calculated in this study) y = 1.3166x + 4.3176 (Chapter 2, Figure 2.6b, Table 2.1), the *M. pyrifera* uptake rate at a substrate concentration of 13.95 µM (x) was 22.69 µmol gdw^{-1} h^{-1} (or 317.64 µg gdw^{-1} h^{-1}). Assuming that the one kilogram of *M. pyrifera* starts off at 1.1% nitrogen (the critical value indicating depletion of nitrogen reserves (Gerard 1982b)), eleven grams of the one kilogram of *M. pyrifera* is nitrogen and 989 grams is other material. As the *M. pyrifera* is subjected to the salmon farm nitrogen input, theoretically, nitrogen uptake will occur and hence there will be a change in the *M. pyrifera* percentage nitrogen content. The increase in percentage nitrogen content after one hour of exposure can be estimated by taking the *M. pyrifera* ammonium uptake rate in µg gdw^{-1} h^{-1} and converting uptake rate to g gdw^{-1} h^{-1} and then multiplying by 989 (the amount of *M. pyrifera* in grams that is not nitrogen). This gives an increase of 0.3141 g of nitrogen. The amount of nitrogen in the

kilogram of *M. pyrifera* is now 11.31 g and the percentage nitrogen content of *M. pyrifera* is theoretically 1.13%.

Assuming that the nitrogen input from the salmon farm remains constant, *M. pyrifera* only takes up salmon farm-derived nitrogen and that the one kilogram of *M. pyrifera* continues to take up nitrogen at the same rate, then it will take 63 hours (2.6 days) to obtain a nitrogen content of at least three percent (Figure 4.2). However, it is unlikely that the uptake of nitrogen will remain constant throughout the 63 hour period. *M. pyrifera* uptake rate can change in response to nutritional history (Haines and Wheeler 1978) and the presence and absence of light (day and night) (Gerard 1982b). Additionally, *M. pyrifera* could release some of its sequestered nitrogen as exudates (*sensu* De Burgh and Fankboner 1978; Abdullah and Fredriksen 2004; Salaün et al. 2012). Nevertheless, these calculations can still indicate the short-term nitrogen enrichment buffering capacity of *M. pyrifera* and illustrate how the nitrogen status of *M. pyrifera* can change given nitrogen inputs from the Kiwa 1 salmon farm and the *M. pyrifera* ammonium uptake rate.

(4.3) How much *M. pyrifera* needs to be cultivated to sequester the Kiwa 1 salmon farm nitrogen inputs?

To determine how much *M. pyrifera* needs to be cultivated to sequester the Kiwa 1 salmon farm nitrogen inputs, it is useful to determine how much salmon farm-derived nitrogen a one hectare standing crop of *M. pyrifera* will sequester during different seasons. The one hectare growing setup is defined as a 100 m x 100 m (10,000 m^2) area with 100 m long surface running (two metres deep) growing lines spaced two metres apart. In total that is 5,000 metres of rope.

Research on *M. pyrifera* aquaculture has shown that cultivated *M. pyrifera* biomass can be between 14-80 kg m^{-1} (wet weight) of rope (Gutierrez et al. 2006; Westermeier et al. 2006; Macchiavello et al. 2010; Correa et al. in press). At approximately a 4:1 wet to dry ratio (ratio determined in this study), *M. pyrifera* biomass in dry weight equates to 3.5-20 kg m^{-1} of rope. Consequently, the biomass of *M. pyrifera* that could be supported on the one hectare rope setup would be between 70 and 400 tonne wet weight or 17.5 and 100 tonne dry weight.

In summer, *M. pyrifera* from the salmon site was on average 2.05% nitrogen (Figure 3.4a). According to the two source isotope mixing model (Table 3.4), during summer, 68% of

nitrogen sequestered by *M. pyrifera* from the salmon site is of pellet (salmon farm) origin. Consequently, whilst the hectare of *M. pyrifera* will take up salmon farm-derived nitrogen, it will simultaneously take up naturally derived nitrogen. Therefore, an *M. pyrifera* individual from the salmon farm site during summer is only 1.4% salmon farm derived nitrogen. In autumn, *M. pyrifera* from the salmon site was on average 2.59% nitrogen (Figure 3.4a). The two source isotope mixing model (Table 3.4) revealed that during autumn, 26% of nitrogen sequestered by the *M. pyrifera* from the salmon site is of salmon farm origin. Therefore, an *M. pyrifera* individual from the salmon farm site during autumn is only 0.67% salmon farm derived nitrogen.

If the *M. pyrifera* cultivation estimate is 3.5 kg m^{-1} of rope, the amount of salmon farm-derived nitrogen sequestered by one hectare of *M. pyrifera* during summer is 17,500 x 0.014 = 245 kg. If the *M. pyrifera* cultivation estimate is 20 kg m^{-1} of rope, the amount of salmon farm-derived nitrogen sequestered by one hectare of *M. pyrifera* is 100,000 x 0.014 = 1,400 kg. If the cultivation estimate of 3.5 kg m^{-1} of rope is applied to autumn *M. pyrifera* percentage nitrogen estimates, the amount of salmon farm-derived nitrogen sequestered by one hectare of *M. pyrifera* is 17500 x 0.0067 = 117.25 kg. If the *M. pyrifera* cultivation estimate is 20 kg m^{-1} of rope, the amount of salmon farm-derived nitrogen sequestered by one hectare of *M. pyrifera* during autumn is 100,000 x 0.0067 = 670 kg.

Using the estimates for the amount of salmon farm-derived nitrogen one hectare of *M. pyrifera* can sequester, the total amount of *M. pyrifera* needed (in hectares) to sequester the seasonal (summer and autumn) salmon farm nitrogen inputs can be determined. The estimated yearly total of Kiwa 1 salmon farm nitrogen inputs is 279,532 kg (calculated from fish excretion and leaching of nitrogen from sediments, section 4.2.1), that is 69,883 kg each season (assuming nitrogen input rates remain constant throughout the year). If one hectare of *M. pyrifera* can sequester 245 kg of nitrogen during summer, the standing crop of *M. pyrifera* needed to sequester the Kiwa 1 nitrogen inputs would be 285 hectares (2.85 km^2, 1,425 km of rope). If one hectare of *M. pyrifera* can sequester 1,400 kg of nitrogen during summer, 50 hectares (0.50 km^2, 250 km of rope) of *M. pyrifera* would be required. Using the autumn estimate for the amount of nitrogen one hectare of *M. pyrifera* can sequester, 117.25 kg, the standing crop of *M. pyrifera* needed to sequester the Kiwa 1 nitrogen inputs would be 596 hectares (5.96 km^2, 2,980 km of rope). If one hectare of *M. pyrifera* can sequester 670 kg of nitrogen during autumn, 104 hectares (1.04 km^2, 520km of rope) of *M. pyrifera* would be required.

The feasibility of IMTA with salmon and *M. pyrifera* in Big Glory Bay will depend on the *M. pyrifera* biomass that can be achieved per metre of rope as well as season. If an optimum *M. pyrifera* biomass (20 kg m^{-1}) could be achieved in summer, 0.5 km^2 of *M. pyrifera* would be required to sequester the salmon farm nitrogen inputs. Such and area is a large portion of Big Glory Bay (surface area of Big Glory Bay is 12 km^2) and it would be unlikely that such a large area would be allocated to *M. pyrifera* cultivation. However, even if 0.25 km^2 (25 hectares) could be allocated to *M. pyrifera* cultivation then theoretically half of the summer Kiwa 1 salmon farm-derived nitrogen inputs could be sequestered. If a low biomass (3.5 kg m^{-1}) of *M. pyrifera* can be achieved in summer then 2.85 km^2 would need to be allocated to *M. pyrifera* cultivation. The required space would thus be around one quarter of the surface area of Big Glory Bay and would require the closure of mussel and oyster farms to make space for *M. pyrifera* cultivation. Because *M. pyrifera* sequesters less salmon farm-derived nitrogen in autumn, the amount of *M. pyrifera* needed to sequester all of the salmon farm nitrogen inputs during autumn is higher and ranges between 1.04 km^2 and 5.96 km^2 (depending on the biomass achieved per metre of rope). Again, the required area is a significant portion of Big Glory Bay and is thus unlikely to be feasible. However, if the optimum *M. pyrifera* biomass could be achieved and 0.25 km^2 of *M. pyrifera* was cultivated in autumn, one quarter of the autumn salmon farm nitrogen inputs could theoretically be sequestered.

These results demonstrate that cultivating *M. pyrifera* with the aim to sequester all of the nitrogen coming from the Kiwa 1 salmon farm is not a feasible option due to space requirements. However, cultivation of *M. pyrifera* with the aim to sequester a portion of the salmon farm-derived nitrogen could still be worthwhile from an ecological and economic perspective. If enough *M. pyrifera* could be cultivated to take up half, or even a quarter of the of the nitrogen coming from the salmon farm during summer, there is the potential for the *M. pyrifera* to contribute significantly to the prevention of harmful algal blooms (HABs) which have been known to occur during this time (Chang et al. 1990, MacKenzie 1991).

(4.4) Conclusions and future research considerations

By combining data from *M. pyrifera in situ* ammonium uptake experiments with data on the theoretical salmon farm nitrogen input rate, it is evident that *M. pyrifera* can be used as a nutrient buffer through its capacity to sequester salmon farm-derived nitrogen. *M. pyrifera* can

reach its maximum three percentage nitrogen content in 63 hours (2.6 days) given the current Kiwa 1 salmon farm nitrogen input rate and the *M. pyrifera* ammonium uptake rate. There is the potential for *M. pyrifera* cultivation to sequester a significant portion of Kiwa 1 salmon farm-derived nitrogen but *M. pyrifera* nitrogen sequestration capacity will depend on the biomass of *M. pyrifera* achievable per metre of rope and season in which the *M. pyrifera* is grown. If an optimum biomass can be achieved (20 kg m^{-1}), the required cultivation area to enable the sequestration of all salmon farm nitrogen would be 0.5 km^2 and 1.04 km^2 in summer and autumn respectively. If a low biomass could be achieved (3.5 kg m^{-1}), the required area of *M. pyrifera* cultivation to enable the sequestration of all salmon farm nitrogen would be 2.85 km^2 and 5.96 km^2 in summer and autumn respectively. Such space requirements be a significant area of Big Glory Bay and would require the closure of mussel and oyster farms. However, even if only a portion of the salmon farm-derived nitrogen could be sequestered by *M. pyrifera* cultivation, the IMTA approach could still be worthwhile. The co-cultured *M. pyrifera* could buffer against eutrophication and associated HABs and provide oxygen, habitat, the potential for economic diversification and a point of difference in the market place.

Future research should generate estimates of *M. pyrifera* biomass that can be supported per metre of rope in Big Glory Bay. This will enable more accurate estimates of required *M. pyrifera* cultivation space to be made. There is also the potential to further develop the models used in this chapter by accounting for more variables including: additional seasons, *M. pyrifera* growth rates and differing diel nitrogen uptake rates, changes in *M. pyrifera* uptake rate with change in nutritional history and hydrodynamic conditions around the farm.
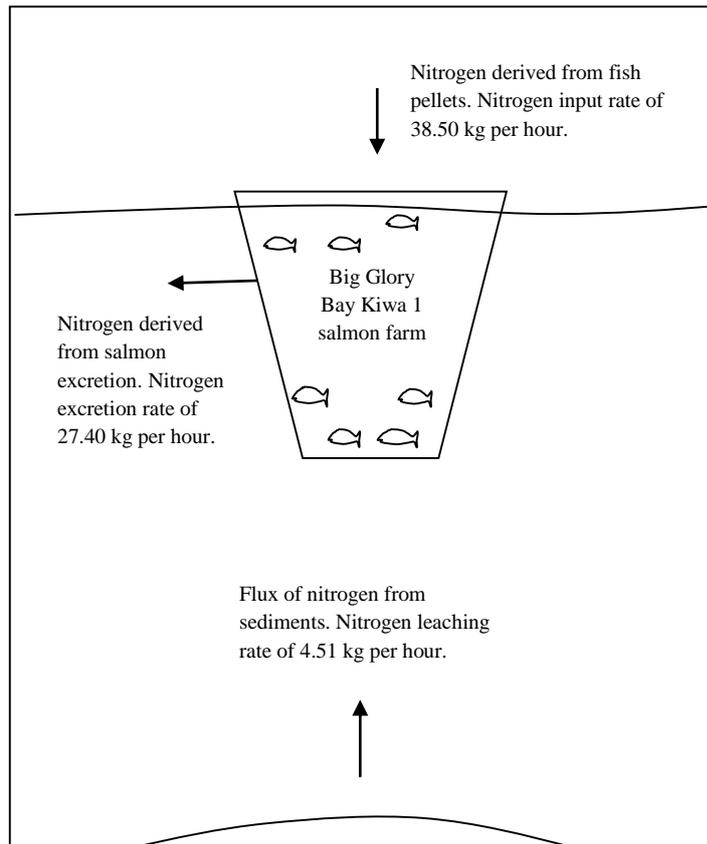
Figure 4.1: Conceptual diagram of the Kiwa 1 salmon farm (Big Glory Bay, Stewart Island, New Zealand) showing sources of salmon farm-derived nitrogen and the amount of nitrogen produced per hour.
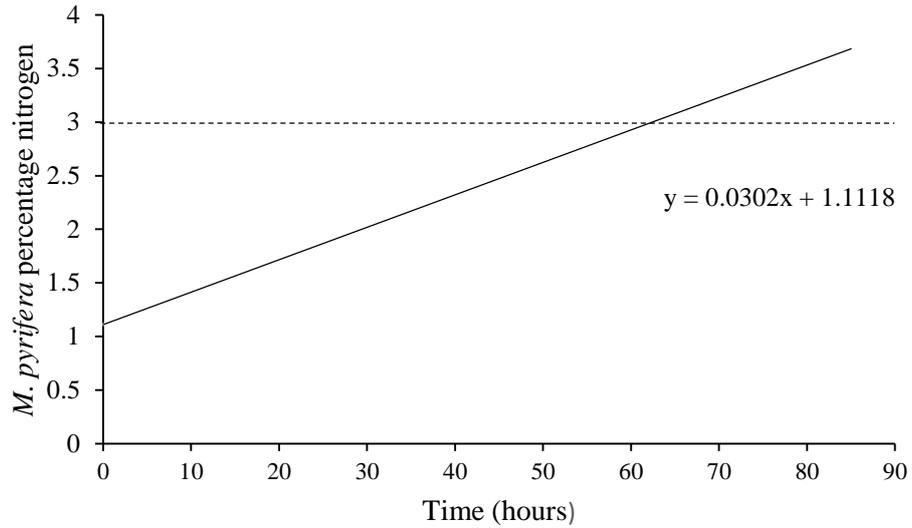
Figure 4.2: Linear regression showing the theoretical change in *M. pyrifera* percentage nitrogen content with time given the nitrogen input rate from the Kiwa 1 salmon farm (Big Glory Bay, Stewart Island, New Zealand) and ammonium uptake rate of Stewart Island *M. pyrifera*. Dashed line outlines the maximum percentage nitrogen content *M. pyrifera* can achieve.

Chapter 5 Conclusions, applications and future work

This thesis set out to (1) determine the *in situ* nitrogen uptake kinetics of *M. pyrifera* and whether tissue excision affected nitrogen uptake and (2) assess the potential for *M. pyrifera* to be used in an IMTA approach with salmon and mussels in Big Glory Bay, Stewart Island, New Zealand. Conclusions from this thesis have applications for future research.

(5.1) *M. pyrifera in situ* nitrogen uptake and effect of tissue excision

In the *in situ* nitrogen uptake experiments, *M. pyrifera* took up ammonium faster than nitrate and displayed rate-unsaturable uptake for ammonium (~80 μM) and uptake discontinuity for nitrate. Nitrate displayed uptake discontinuity with rate-saturable uptake initially ($V_{max} = 31.67$ μmol gdw^{-1}h^{-1}, $K_s = 61.00$ μM) and then uptake increased linearly thereafter. Excision of the *M. pyrifera* blade did not significantly affect the uptake of ammonium. It was deduced that a long tissue recovery time (at least eighteen hours) allowed the excised tissue to recover to a degree that ammonium uptake was unimpaired and that that a short incubation period (one hour) meant that loss of the capacity for translocation was not a significant factor influencing uptake. These results suggest that *in vitro* uptake experiments which utilise excised macroalgal tissue and a short incubation time may still be able to produce useful and accurate data despite a reduced capacity for translocation and damage to the tissue used. In future nutrient uptake experiments where excised macroalgal tissue is used, preliminary experiments should be conducted to determine a suitable recovery period for the studied species.

(5.2) *M. pyrifera* as an IMTA species in Big Glory Bay

Results from this study suggest that *M. pyrifera* could be successfully used in an IMTA approach with salmon in Big Glory Bay, Stewart Island, New Zealand. During the summer period, *M. pyrifera* co-cultured with salmon displayed some evidence of improved growth rate and had higher nitrogen content and soluble ammonium concentrations in their tissues. In autumn however, there was evidence to suggest that increasing naturally occurring seawater nitrogen concentrations negated the effect of salmon farm nitrogen provision on *M. pyrifera* growth rate and nitrogen status. Analysis of *M. pyrifera* δ¹⁵N signatures suggested that on

average 68% of assimilated nitrogen was derived from the nitrogen in salmon pellets during summer. In autumn however, evidence indicated that salmon farm-derived nitrogen was a less important source of nitrogen and $\delta^{15}N$ signatures suggested that on average only 26% of assimilated nitrogen was derived from the nitrogen in salmon pellets. The results from the IMTA trials in Big Glory Bay show that *M. pyrifera* can take up salmon farm-derived nitrogen but seasonal differences in nitrogen demand suggest that the potential for nitrogen sequestration by this species will be greatest during the summer period. The co-culture of mussels with *M. pyrifera* in Big Glory Bay does not appear to be a viable IMTA option as there was limited evidence of mussel farm-derived nitrogen sequestration by *M. pyrifera*.

After accounting for *M. pyrifera in situ* nitrogen uptake kinetics, Big Glory Bay seawater chemistry and *M. pyrifera* nitrogen status data, the short-term buffering capacity of *M. pyrifera* was determined with respect to salmon farm nitrogen inputs. Given the Big Glory Bay nitrogen input rate of 31.91 kg h^{-1}, co-cultured *M. pyrifera* can theoretically reach their maximum three percent tissue nitrogen content in 63 hours. Given the nitrogen input rate and nitrogen content of the *M. pyrifera* from the salmon site, the nitrogen sequestration potential of one hectare of *M. pyrifera* is between 245 kg and 1,400 kg in summer and 117.25 kg and 670 kg in autumn. If the aim of IMTA in Big Glory Bay is to sequester all of the nitrogen coming from the Big Glory Bay salmon farm, the required amount of *M. pyrifera* would be between 50 hectares (0.5 km^2) and 596 hectares (5.96 km^2) depending on season and the biomass of *M. pyrifera* achievable per metre of rope. The area required would thus be a significant portion of Big Glory Bay (12 km^2). However, even if a portion of the nitrogen from the Kiwa 1 salmon farm could be sequestered by *M. pyrifera* cultivation, the IMTA approach may be worthwhile. The co-cultured *M. pyrifera* could buffer against eutrophication and associated HABs particularly during the summer when HABs have historically been a problem and *M. pyrifera* cultivation could provide oxygen, habitat, the potential for economic diversification and a point of difference in the market place.

(5.3) Future IMTA research

Limited IMTA research has been performed in New Zealand and hence future research should explore the possibility of co-culturing different species of macroalgae with salmon to try and determine the most appropriate IMTA species for both nutrient sequestration and economic

gain. Larger scale experiments with *M. pyrifera* over a longer time period are also warranted to further determine the potential for IMTA in Big Glory Bay and to assess the economic viability of such an approach. Future *M. pyrifera* nutrient uptake models should account for more variables including: season, *M. pyrifera* growth rates and differing diel nutrient uptake rates, changes in *M. pyrifera* uptake rate with change in nutritional history and hydrodynamic conditions around the farm. It would also be beneficial to improve the accuracy of the salmon farm nitrogen input rate to enable better estimates of *M. pyrifera* nitrogen uptake rate and sequestration capacity.

(5.4) The future of IMTA in New Zealand

As the New Zealand aquaculture industry grows and intensifies to meet the New Zealand Government's goal of a $1 billion aquaculture industry by 2025, the feasibility of IMTA will need to be tested and implemented if aquaculture in New Zealand is to expand sustainably. The government and aquaculture industry will need to seriously consider IMTA as an option to both help combat the problems (HABs and anoxic conditions) associated with the intensification of aquaculture as well as the potential for IMTA to contribute to the economic growth and diversification of the aquaculture industry. *M. pyrifera* is an ideal species for the New Zealand aquaculture industry to consider for use in IMTA with its potential being greatest during the summer period when the risk of HABs and anoxic conditions are highest. The New Zealand aquaculture industry has ten years to reach its $1 billion dollar goal which is a sufficient period to investigate and implement IMTA in New Zealand. Only time will reveal whether the New Zealand aquaculture industry will embrace sustainable growth though IMTA or will continue with the status quo and hence progress in a less sustainable manner.

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