Gracilaria transtasmanica: a potential novel target species for the bioremediation of nutrient enriched estuaries

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Abstract

Estuaries are increasingly becoming eutrophic due to anthropogenically induced nutrient enrichment. Presently, 24% of global anthropogenically derived nutrients released into coastal catchments are delivered directly into coastal waters such as estuaries. Thirty five percent of New Zealand's estuaries are susceptible to eutrophication and some are already severely degraded. In-situ macroalgal bioremediation – the cultivation of live macroalgae directly in nutrient-enriched waters for the primary purpose of nutrient assimilation – could help prevent and reduce estuarine eutrophication.

The indigenous red macroalga *Gracilaria transtasmanica* has been identified as a potential target species for bioremediation in New Zealand estuaries. Samples of *G. transtasmanica* were collected from estuaries in the Bay of Plenty region of New Zealand, and were isolated, formally identified and scaled up into stock cultures. Controlled experiments were then used to assess the bioremediation potential of this species in winter and summer conditions under ambient and extreme nutrient and sediment levels. Biomass productivity, photosynthetic health, and N removal were assessed to quantify performance. Biomass productivity and total N removed were 45 and 50% higher respectively in summer compared to winter. Biomass productivity ranged from 0.4 – 4.3 g DW m^2 in summer and 0.6 – 1.8 g DW m^2 in winter, and total N removed ranged from 6.0 – 30.7 mg N in summer and 6.1 – 9.1 mg N in winter. In contrast, standardised N removal rates were higher in winter (37.8 mg g DW biomass $growth^{-1}$ ± 0.6 S.E.) compared to summer (22.3 mg g DW biomass $growth^{-1}$ ± 1.2 S.E.), suggesting that the biomass was storing N and therefore that N uptake was independent of productivity under sub-optimal growing conditions. Productivity was significantly affected by sediment treatment, however this effect differed seasonally, with higher productivity in the high

sediment treatments in summer, and lower productivity in the high sediment treatments in winter. These results demonstrate that *G. transtasmanica* can assimilate nutrients under a range of environmental conditions that are representative of both local background and high levels of suspended sediment and nutrient concentrations, and therefore is a viable target species for the bioremediation of nutrient enriched estuaries.

In addition to nutrient uptake and productivity, target species for in-situ estuarine bioremediation need to maintain productivity under a range of challenging abiotic conditions. In chapter three of this thesis, a series of independent experiments assessed the tolerance of *G. transtasmanica* to ambient and extreme levels of salinity, exposure, and light limitation that are likely to be experienced in an estuary. Photosynthetic functioning and growth were used to quantify the tolerance range of *G. transtasmanica* in each experiment. Specific Growth Rate (SGR) was significantly affected by salinity, exposure, and light limitation. *Gracilaria transtasmanica* was able to grow in salinities ranging from 5 to 35 ppt, but growth rates decreased with decreasing salinity. Exposure periods of up to 9 hours were tolerated, but growth rates were decreased as exposure period increased. *Gracilaria transtasmanica* was able to maintain growth with a loss of up to 75% of ambient light and was also able to tolerate short periods of no light. Measurements of optimal quantum yields showed photosynthetic function was unaffected by salinity, exposure, or light limitation. These results demonstrate the high tolerance and adaptability of *G. transtasmanica* to a broad range of challenging abiotic estuarine conditions.

In combination, these findings demonstrate that *Gracilaria transtasmanica* is well suited to estuarine conditions. However, selection of habitats with optimal conditions for growth will maximise productivity. Moreover, as nutrient uptake appears to be independent of

growth under challenging conditions, low productivity may not result in reduced bioremediation capability. These characteristics make *G. transtasmanica* an ideal species for in-situ bioremediation in New Zealand estuaries. Additionally, *G. transtasmanica* biomass is suitable for use in a range of bioproducts. Therefore, there is potential for a bioremediation based circular economy approach with the use of harvested *G. transtasmanica* biomass following bioremediation. In-situ trials are required to verifying the findings reported here and enable optimisation of both bioremediation efficiency and biomass productivity.

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Glossary

List of terms & definitions

Term	Definition/s (for the purpose of this thesis)	Source/s
Estuary	A partially enclosed coastal water body in which freshwater runoff, often seasonally and episodically pulsed dilutes salty ocean water, the biotic structure is influenced by dynamic tidal action and function	Flemer and Champ (2006)
Anthropogenic	Caused by human beings"; anthropic.	Merriam-Webster (2022a)
Eutrophication	The process by which a body of water becomes enriched with nutrients that stimulate excessive primary production, OR " An increase in the rate of supply of organic matter (OM) to an ecosystem".	Pinckney *et al.* (2001)
Turbidity	A measure of the degree to which the water loses its transparency due to the presence of suspended particulates causing scattering of light. E.g. optical clarity. Can also be a measure of the amount of suspended solids in the liquid.	Kitchener *et al.* (2017)
Sedimentation	The deposition and/or accumulation of sediment (for example "increased sedimentation" = additional sediment accumulation on the benthos).	Booth (2020); Collins (2022)
Environmental degradation	A change and/or disturbance to the environment which is perceived to be deleterious or undesirable. A shift away from a "natural environment".	Johnson *et al.* (1997)
Natural Environment	An environment relatively unchanged or undisturbed by human culture, e.g. existing in or derived from nature; not made or caused by humankind	Johnson *et al.* (1997)
Terrigenous	Being or relating to oceanic sediment derived directly from the destruction of rocks on the earth's surface	Merriam-Webster (2022b)
Ash	The fraction of mineral ash left after burning organic matter	Vassilev *et al.* (2017)

List of abbreviation's

Abbreviation	Translation	Description
FW	Fresh Weight	Weight of drained and spun fresh biomass
DW	Dry Weight	Weight of biomass after oven or freeze-drying
FW:DW	Fresh weight to dry weight ratio	Calculated to provide insight into the water content of biomass
AFDW	Ash Free Dry Weight	Calculated biomass weight minus known ash (see terms above) content
PAM	Pulse Amplitude Modulation	Process of measuring photosynthetic performance in biomass tissue
PSII	Photosystem two (II)	The first protein complex in the light-dependent reactions of oxygenic photosynthesis
Y (II)	Effective quantum yield	Performance of photosystem II – low Y(II) values show short term cell damage
F_v/F_m	Optimal quantum yield	Performance of photosystem II – low F_v/F_m values show long term cell damage
N	Nitrogen	Naturally occurring chemical element
P	Phosphorus	Naturally occurring chemical element
SGR	Specific Growth Rate	Calculated with the equation $Ln(Bf/Bi)/T * 100$: where Bf and Bi = the final and initial biomass (g DW) and T is the number of days in culture

Chapter 1
General introduction & literature review

1.1 Estuarine degradation

Estuarine habitats are experiencing ecological degradation at an unprecedented rate and loss of coastal ecosystem services is now globally widespread (Figure 1.1, Malone & Newton, 2020). In some cases, entire coastal regions are heavily degraded and rated as highly impacted areas, including the Baltic Sea, the Northern Adriatic Sea, the Gulf of Mexico, the East China Sea, and the Great Barrier Reef (Malone & Newton, 2020). For example, 176 out of 189 sites surveyed in the Baltic Sea were affected by eutrophication (Andersen *et al.*, 2011), whilst the Gulf of Mexico is experiencing extensive toxic algal blooms across multiple coastal ecosystems (Ulloa *et al.*, 2017). In the United States of America, 65% of estuarine habitats are currently categorised as moderately to severely eutrophic (Bricker *et al.*, 2008). However, more recent estimates have suggested this could in fact be as much as 78% (Malone & Newton, 2020). Similarly, 66% of estuaries in Europe do not meet "good ecological status" (Malone & Newton, 2020). The major contributors to estuarine degradation are nutrient enrichment – causing eutrophication - and increased turbidity caused by sedimentation. The primary cause of nutrient enrichment and sedimentation is anthropogenic land use (Kennish, 2002). As the human population has doubled in the last 50 years (OurWorldData, 2020), environmental pressures have correspondingly increased (Kennish, 2002; Flemer & Champ, 2006). Furthermore, the human population is largely based in or close to coastal zones (Harris *et al.*, 2016). As of 2017, 40% of the world's population lived within 100km of the coast; with nearly 70% of

the world's largest cities situated on the shores of estuaries (Harris *et al.*, 2016). It is therefore not surprising that estuaries have degraded significantly over the past 50 years. Furthermore, as continued population growth creates an increasing demand for food, leading to further terrestrial modification, the rates of global estuarine degradation are likely to escalate (Flemer & Champ, 2006).

Figure 1.1: Distribution of eutrophic coastal ecosystems (indicated by red dots) as categorised by Malone and Newton (2020). Note only coastal ecosystems surveyed and categorised are displayed here.

Estuarine degradation is also an increasing concern in New Zealand. Up to 35% of estuaries in New Zealand are considered susceptible to eutrophication (Plew *et al.*, 2018a). This susceptibility is linked to the physical attributes of the estuary catchment land-use patterns, and macroalgal presence (Plew *et al.*, 2020). A key contributor to estuarine eutrophication in New Zealand is agricultural and horticultural production, which utilizes 58% of New Zealand's land cover (Figure 1.2) and has caused elevated nutrient and sediment loads in coastal ecosystems (MacLeod *et al.*, 2008; Sunda & Cai, 2012; Plew *et al.*, 2018a; Mangan

et al., 2020a). Regions and catchments with intensive anthropogenic land use are considerably more suspectable to eutrophication than less developed areas (Plew *et al.*, 2020). For example, the New River estuary in the Southland region is showing substantial degradation and continuing loss of ecosystem health due to eutrophication and pollution caused by rapid and extensive land use shifts from predominantly native forest to orchid plantations and dairy pasture (McDowell & Wilcock, 2008; Chobtang *et al.*, 2016; Brown, 2019). Urban nitrogen sources are also contributors to eutrophication at an increasing rate (Chakravarthy *et al.*, 2019) largely due to 86% of New Zealanders living in less than 1% of the land area, causing isolated and intensified impacts (Chakravarthy *et al.*, 2019). Waterways in these urban areas are showing significant nutrient enrichment with urban streams having as much as 19.5 times the level of nitrogen compared to streams with natural bush cover (Chakravarthy *et al.*, 2019). Therefore, estuaries in regions or catchments with intensive agricultural activity or large human populations are more likely to become eutrophic. This may be further exacerbated if the estuary has a large intertidal area, long tidal flushing time, and is already prone to macroalgal blooms (Nelson *et al.*, 2015; Plew *et al.*, 2020).

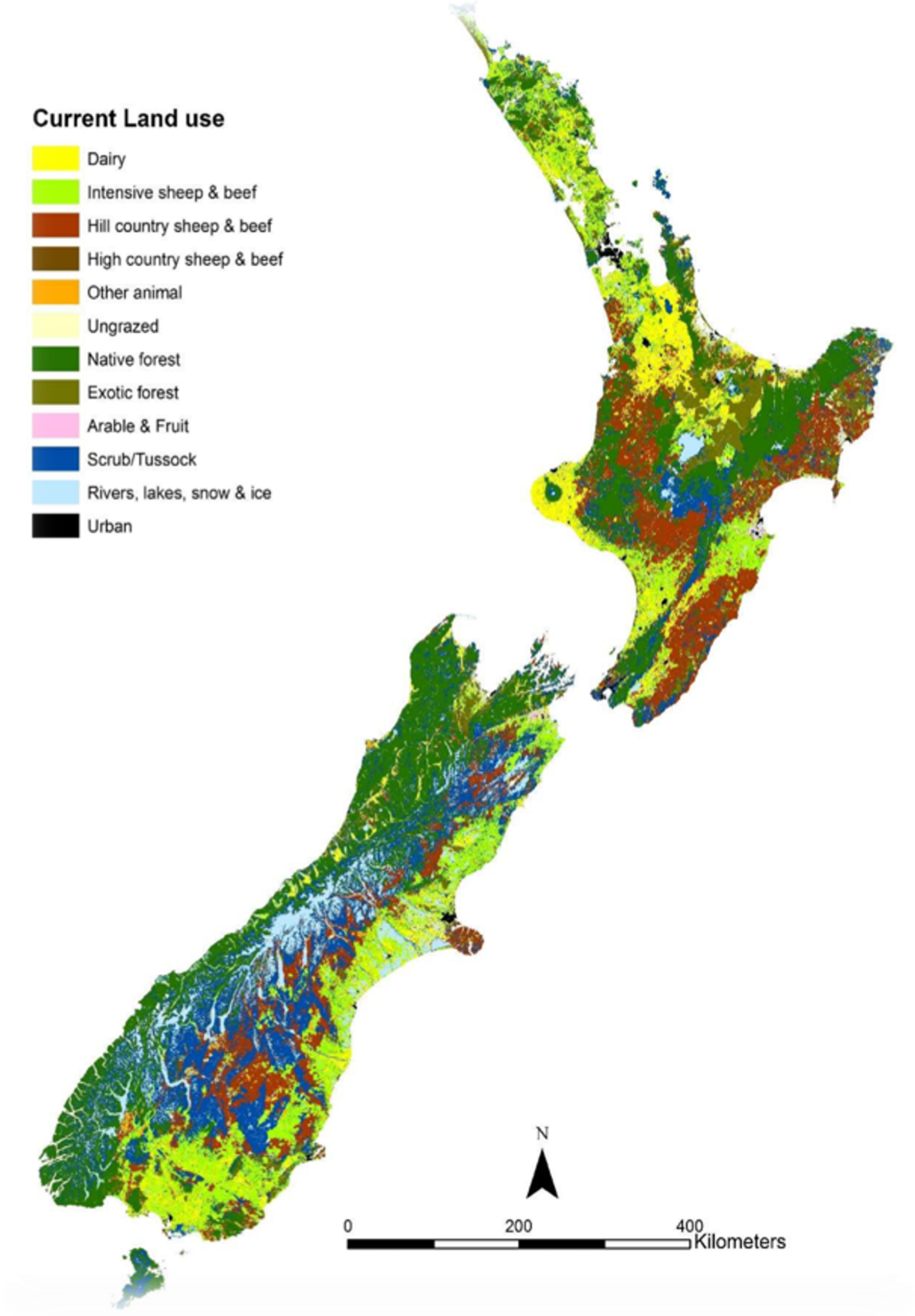

Figure 1.2: Present land use in New Zealand, showing catchment development and intensification. (Plew *et al.*, 2018a).

1.1.1 Coastal Eutrophication

Eutrophication is the leading cause of estuarine degradation both globally and in New Zealand (Plew *et al.*, 2018a; Malone & Newton, 2020). Eutrophication is the depletion of oxygen within the water, caused by the respiration and decomposition of primary producers that have grown at elevated levels due to nutrient enrichment (Pinckney *et al.*, 2001). Coastal eutrophication can have substantial negative impacts, including habitat loss and declines in biodiversity (Pinckney *et al.*, 2001; Bricker *et al.*, 2008; Malone & Newton, 2020). Habitat loss is largely caused by the depletion of oxygen in the environment leading to hypoxia and anoxia to toxic extremes (Ellis *et al.*, 2000; Bricker *et al.*, 2008). Biodiversity loss results when key species carrying out biogeochemical cycling and bioturbation become subject to lethal and sub-lethal conditions as the ecosystem degrades (Gray *et al.*, 1999; Ellis *et al.*, 2000). Although estuaries are naturally resilient and dynamic systems, the multiple stressors they are frequently subject to, introduced by a range of anthropogenic activities, can reduce natural resilience and weaken biological community structure (Thrush *et al.*, 2003; Thrush *et al.*, 2013; O'Meara *et al.*, 2017; Malone & Newton, 2020). With continued anthropogenic stressors, estuaries can exhibit a sudden shift in function and structure from a diverse system resilient to short-term impacts, to one that is dominated by a few species that can capitalize on highly specialist conditions, e.g., carbon-rich and hypoxic (Pinckney *et al.*, 2001; Bricker *et al.*, 2008; Hewitt & Thrush, 2019). This ecological shift, known as a tipping point, causes the system to become unable to carry out key processes such as nutrient cycling, with implications for ecosystem services (Appendix 1.1, Ellis *et al.*, 2000; Hewitt & Thrush, 2019; O'Higgins *et al.*, 2020).

Harmful algal blooms (HABs) are one of the most prominent symptoms of eutrophication in estuarine habitats (Hallegraeff, 2003; Nelson *et al.*, 2015; Plew *et al.*, 2018a). Macroalgal blooms are also used as indicators of the degree of eutrophication in an estuary (Plew *et al.*, 2018a). Estuaries are one of the most productive habitats on the globe, largely led by algal growth at the base of the food web (Correll, 1978; Statham, 2012). However, algal species dynamics can shift under nutrient enriched conditions, resulting in a "bloom" of unwanted toxic microalgae species (Glibert, 2017). If these conditions are maintained, the estuary will shift to an algal dominated and highly nutrient-enriched system, and therefore become eutrophic by definition (Figure 1.3, Bricker *et al.*, 2008).

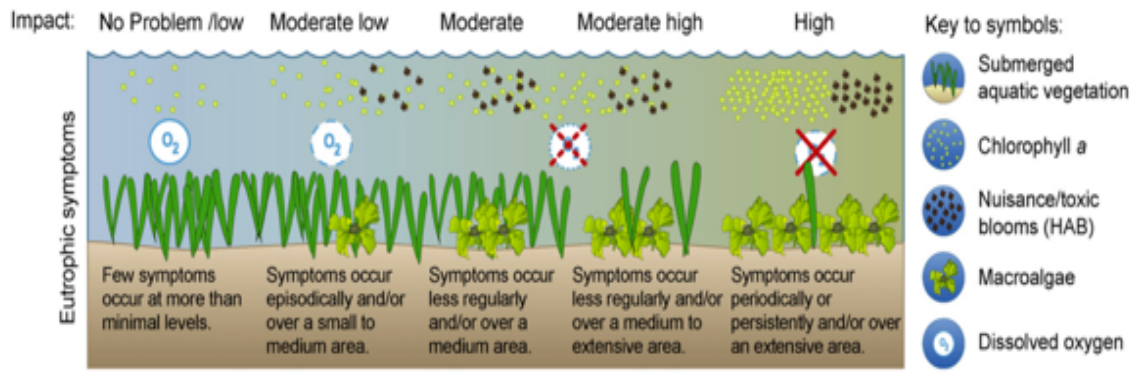

Figure 1.3: Depiction of the progression of eutrophication leading to ecological shifts in species diversity and biomass over time. Adapted from: Bricker *et al.* (2008).

1.1.1.1 Sources of nutrients

Nutrient delivery to coastal waters has multiple pathways including diffuse sources such as pasture runoff and submarine groundwater discharge (see below), and point sources such as storm water drains and sewage outfalls. Generally, the most significant contributor of N inputs to coastal ecosystems globally is river runoff, elevated by agricultural pasture runoff, point source discharge, and atmospheric processes (Figure 1.4, Statham, 2012; Malone &

Newton, 2020). Terrigenous nutrient transport via surface waters such as rivers and streams to coastal waters is irregular as it is dependent on regionally variable climate and land use (Kennish, 2002; Howarth, 2008; Statham, 2012; Glibert, 2017; Malone & Newton, 2020). In the last century, riverine nutrient inputs to coastal ecosystems have almost doubled from ~ 27 x 10^9 kg N yr^{-1} to ~ 48 x 10^9 kg N yr^{-1} (Dentener *et al.*, 2006; Howarth, 2008; Duce *et al.*, 2008; Statham, 2012). However, the relative contributions from different sources of N inputs to coastal ecosystems differ with location and are predominantly driven by geology and catchment use. For example, 90% of nutrients transported into the lagoon formed by the Great Barrier Reef are derived from point source discharges into rivers (Malone & Newton, 2020). In contrast, 80% of N and 93% of P delivered into the Northern Adriatic Sea are derived from diffuse sources (Malone & Newton, 2020). Globally, anthropogenic inputs of nutrients into water ways from diffuse sources are considerably greater than anthropogenic point source inputs at ~ 200 x 10^9 kg N yr^{-1} and ~ 45 x 10^9 kg N yr^{-1} respectively (Dentener *et al.*, 2006; Duce *et al.*, 2008; Malone & Newton, 2020). Atmospheric processes also contribute considerable amounts of N to coastal waters through deposition of particles and precipitation (Duce *et al.*, 2008), with an estimated 14% of N inputs to marine environments delivered from the atmosphere (Dentener *et al.*, 2006; Duce *et al.*, 2008; Malone & Newton, 2020). Like river inputs, atmospheric N inputs have risen considerably in the twentieth century from ~ 22 x 10^9 kg N yr^{-1} to > 45 x 10^9 kg N yr^{-1}. Precipitation contributes to N enrichment and has the potential to increase if the global climate continues to destabilise (Michener *et al.*, 1997).

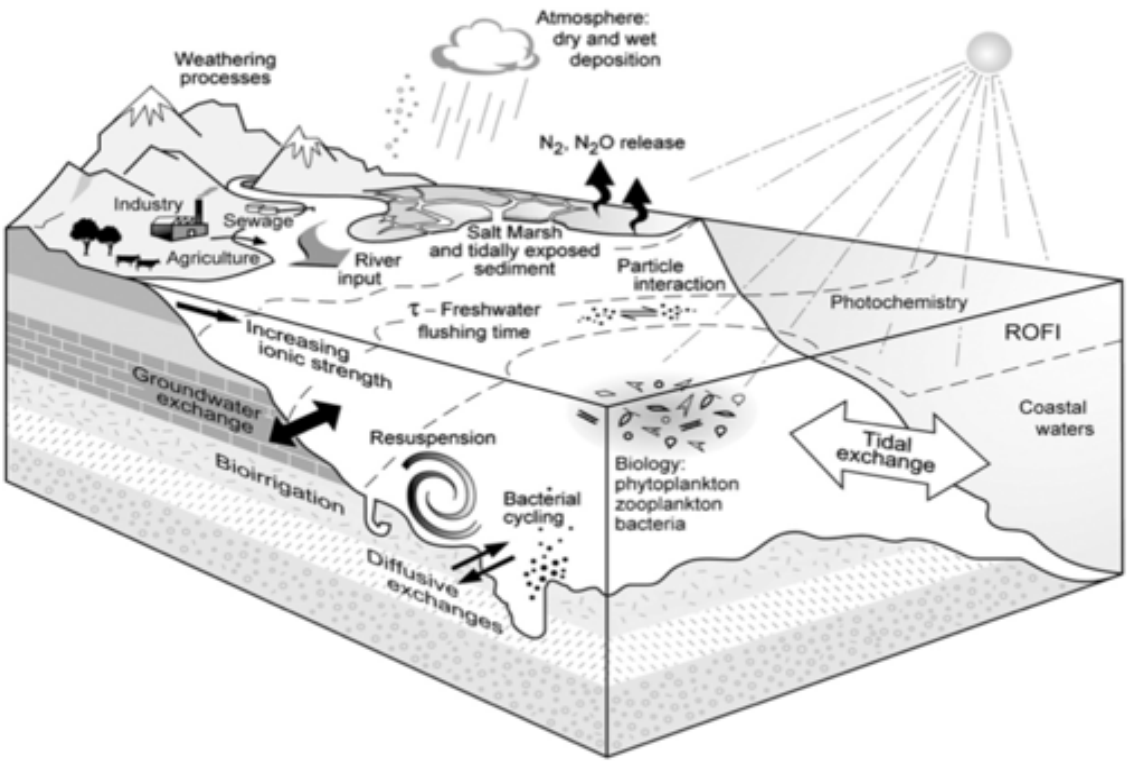

Figure 1.4: Physical and chemical processes, inputs, and exchanges within estuarine systems. Note, ROFI = Region Of Freshwater Influence. Produced by Statham (2012)

Anthropogenic activities are largely considered the most significant source of excess nutrients to coastal ecosystems. Relative to forested land, agriculture and horticulture have significantly higher nutrient runoff to freshwater systems, and subsequently coastal ecosystems, through land clearing and high rates of synthetic fertiliser application (Howarth, 2008; Glibert, 2017; Malone & Newton, 2020). The amount of N delivered varies depending on the primary land use in the catchment (Journeaux *et al.*, 2017), with dairy farming and vegetable cropping having the highest rates of N leaching in New Zealand (Table 1.1). The crop type can affect nitrogen levels due to fixation of atmospheric N by certain cropping legume species such as soy, leading to increased loads of N in the soil that may subsequently leach into waterways (Glibert, 2017; Malone & Newton, 2020). In addition, soil type also influences the amount of nutrient loss. For example, as much as

80% of the nitrogen applied to crops in sandy soils is lost to groundwater and runoff (Howarth, 2008), whilst finer soils leach considerably less N on average (Panagopoulos *et al.*, 2007). Organic matter further contributes to nutrient inputs on larger time scales as it breaks down through microbial pathways into dissolved inorganic forms of nutrients (Bernhard, 2010; Wolanski & McLusky, 2011). Therefore, activities such as the harvesting of forestry plantations which cause considerable pulses of organic matter that is exported to waterways can elevate N inputs over time (Gene *et al.*, 1970). For example, stream nitrate levels in White Mountain National Forest, New Hampshire, increased as much as 56-fold two years after forest harvest (Gene *et al.*, 1970). In addition to agricultural and horticultural activities, human sewage also produces a considerable amount of N and P per year. This sewage is generally captured and processed to some degree, thereby reducing the N and P content, but remaining a point source contributor of Nutrients (Carey & Migliaccio, 2009; Holeton *et al.*, 2011). However, the processed sewage is ultimately discharged, therefore remaining a contributor of N and P to surrounding environments (Loe, 2012; Neveux *et al.*, 2018; Chakravarthy *et al.*, 2019). In Canterbury, New Zealand, N and P from consented sewage effluent discharges is estimated at 550 and 207 tonnes per year, respectively (Loe, 2012).

Table 1.1: Indicative nitrogen leaching figures across various land use type in New Zealand (Journeaux *et al.*, 2017). Note actual leaching figures differ due to individual catchment characteristics and land management practices

Source	N Leaching (Range) kg N/ha/yr
Dairy Farming	20 – 150
Sheep and Beef Farming	6 – 50
Viticulture	5 – 10
Pip fruit	5 – 20
Kiwifruit	10 – 40
Arable cropping	20 – 40
Vegetable cropping	20 – 150
Forestry	2.5 – 4

Submarine groundwater discharge can be another significant source of nutrients in some estuaries (Figure 1.5, Burnett *et al.*, 2006). Estimates suggest this nutrient pathway matches or exceeds the N delivery of surface freshwater surfaces in some locations, with submarine groundwater discharge contribution equalling as much as 80 to 160% of riverine inputs in the North Atlantic Ocean (Statham, 2012). Similarly, some 50% of nitrates in Great South Bay, New York, are sourced from submarine groundwater discharge (Burnett *et al.*, 2006). Submarine groundwater discharge was also found to contribute significantly more nutrients than riverine input in Tauranga Harbour (the one of the largest natural harbours in New Zealand), with submarine groundwater discharge contributing five times the N, and eight times the P input of riverine discharge (Stewart *et al.*, 2018). Sources of nutrients that cause enrichment of diffuse submarine groundwater discharges are often unclear, though they are thought to include poor septic tank management and seepage from agricultural, horticultural, and cropping land (Burnett *et al.*, 2006; Howarth, 2008). Additionally, nutrients can accumulate over time and remain within soils (known as legacy nutrients) and subsequently leach into groundwater aquifers for years and even decades, thereby also actively contributing to nutrient loads in submarine groundwater discharge (Burnett *et al.*, 2006; Ator & Denver, 2015).

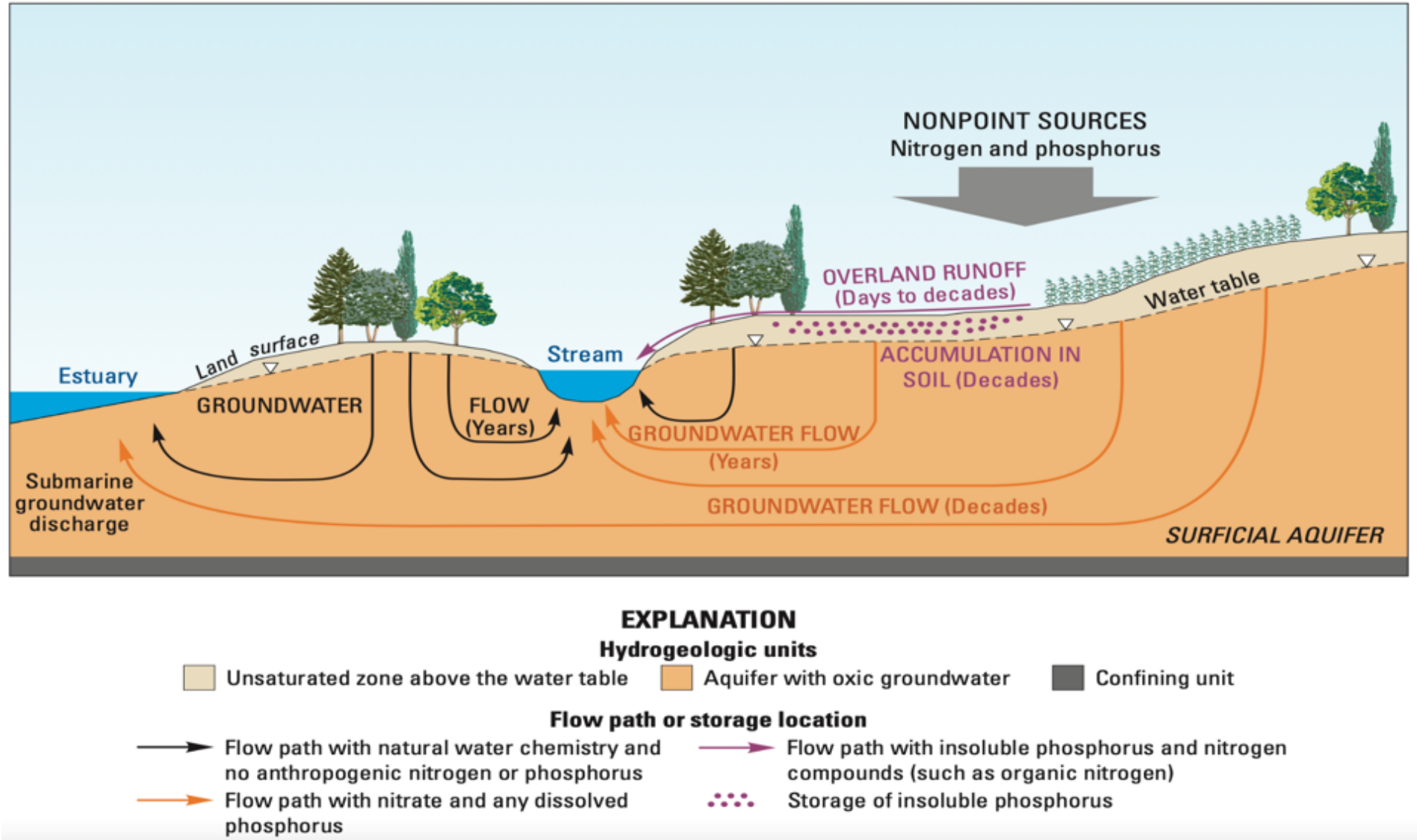

Figure 1.5: Conceptual representation of the anthropogenic influence, and biogeochemical and hydrological processes affecting nitrogen transport dynamics at a catchment scale. Sourced from Ator and Denver (2015)

1.1.1.2 Critical nutrients

The nutrients that cause eutrophication (N and P) are also critical for primary production. N and P have multiple forms, as briefly outlined in Table 1.2. Only those forms available to photosynthetic organisms (DRP & DIN), and therefore direct contributors to eutrophication, are discussed here in detail.

Table 1.2: Forms of nitrogen and phosphorus, and their chemical symbols and associated availability to primary producers.

Form	Chemical symbol	Availability and use
Molecular (atmospheric) nitrogen	N_2	Molecular nitrogen is not available to most plants as it is in a gaseous form. However, through nitrification by bacteria and some algae (cyanobacteria) or lightning, N_2 can be taken into the soil or sediments and made available to photosynthetic organisms. *Note a few plants are capable of nitrifying without bacteria.
Ammonia	NH_{3-}	Ammonia is produced through the conversion of N_2 into a form available to primary producers.
Nitrite	NO_2^-	Nitrite is an interim form of N produced through aerobic bacterial processes converting ammonia to nitrite before further bacterial nitrification convert nitrite to nitrate.
Nitrate	NO_3^-	The form most utilised by plants and other photosynthetic organisms, this form is also easily lost from soil and into groundwater.
Ammonium	NH^+_4	Available for photosynthetic organisms, used directly to produce proteins.
Total nitrogen	TN	Includes all forms of nitrogen, frequently used as a measure of N in an environment, e.g. soil and water testing.
Dissolved inorganic nitrogen	DIN	DIN is available to photosynthetic organisms. DIN can be defined as the sum of inorganic, biologically available nitrogen, including nitrite (NO_2^-), nitrates (NO_3^-), ammonium (NH^+_4), and ammonia (NH_{3-}).
Total Phosphorus	TP	Includes all forms of phosphorus, used as a measure of P in an environment, e.g. soil and water testing.
Dissolved reactive Phosphorus	DRP	The fraction of phosphorus that is available for assimilation by photosynthetic organisms, essentially all forms of PO4 including $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}.

Dissolved Inorganic Nitrogen

Dissolved inorganic nitrogen (DIN) is the sum of inorganic biologically available nitrogen, including nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH^+_4), and ammonia (NH_{3-}). This fraction of TN includes N converted from organic forms via nitrification through a range of bacterial processes in the nitrogen cycle (Figure 1.6; Bernhard, 2010; Wolanski & McLusky, 2011; O'Brien *et al.*, 2016). The majority of processes in the N cycle are carried out by prokaryotes, particularly Nitrosomonas, however eukaryotes and even cyanobacteria also contribute to N transformation (Howarth *et al.*, 1999; Bernhard, 2010; Dos Santos *et al.*, 2012). Oxygen availability largely dictates N transformations as much of the N cycle occurs only anaerobically (Wolanski & McLusky, 2011). Conversely, many of the organisms that assimilate DIN either require and/or produce oxygen. (Wolanski & McLusky, 2011). Uptake rates are also reliant on temperature, light, and species composition (Wolanski & McLusky, 2011). Assimilation of DIN by primary producers is generally an active process, unless ambient N concentration is extremely high (Wolanski & Spagnol, 2000). Once inside the cells of an assimilating organism, NO_2^- and NO_3^- are converted by enzymes into ammonia acids. However, only species specialised in assimilating these forms of N and can produce these enzymes and can derive energy from NO_2^- and NO_3^- (Wolanski & McLusky, 2011; Dos Santos *et al.*, 2012). In contrast, NH^+_4 can be assimilated by both primary producers and bacteria; with bacteria typically being dominant in light-limited, turbid environments, such as under winter conditions or potentially year-round in highly turbid zones (Wolanski & McLusky, 2011). NH^+_4 is the most efficient form of N for macroalgae to assimilate as no further chemical

transformation is required for metabolic use (Wolanski & McLusky, 2011). NH^{+}_{4} also tends to make up the majority of N in coastal areas and estuaries, however, this can change with upwelling and pollution, with which NO_3^- becomes predominant (Wolanski & Spagnol, 2000; Wilkerson & Dugdale, 2008).

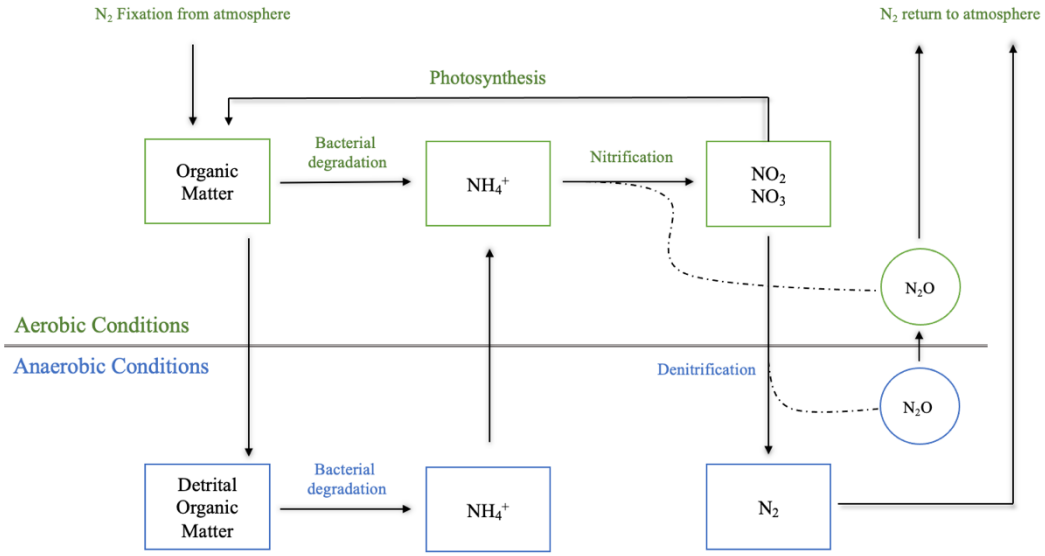

Figure 1.6: Basic processes and conversions occurring within the nitrogen cycle, in both aerobic and anaerobic conditions. Key processes are fixation, nitrification, denitrification, and photosynthesis. Adapted from: UoM (University of Michigan, 2012); O'Brien *et al.* (2016).

Dissolved Reactive Phosphorus

Organic phosphorus (phosphate ion P_{org}) is the most common form of P but is sediment-bound and therefore unavailable to primary producers (Vadas *et al.*, 2007). Dissolved Reactive Phosphorus (PO_4^{3-}) is the form that is biologically available and also essential to photosynthetic organisms. DRP availability involves both biotic and abiotic processes and complex biogeochemical interactions in soil, sediment, and mineral components of the environment (Queiroz *et al.*, 2021). However, there is little in-depth knowledge of the redox cycle and phosphorus/iron relationships which are thought to directly affect interactions and retention of P,

particularly within estuarine environments (Queiroz *et al.*, 2021). The land-based phosphorus cycle is relatively slow, taking at least a year to complete, compared to the nitrogen and biological carbon cycles which may take as little as a few days to a few weeks to complete. For example, it can take as long as 14 months for 60% of P from manure to leach out from soil via rainwater wash-off and seepage (Vadas *et al.*, 2007). Therefore, despite increased inputs of phosphates to aquatic environments via fertilisers, livestock waste, and various anthropogenic products such as some household soaps, both cycling, and transport of P are often rate-limiting for photosynthetic organisms (Queiroz *et al.*, 2021).

1.2 Eutrophication management strategies

Eutrophication mitigation methods are generally categorized into three classes (Table 1.3): land-based treatments used to capture, reduce and dispose of nutrients at the source such as pond treatment; capture of contaminants, such as wetlands and riparian zones; and bottom-of-catchment methods that treat contaminants in receiving waters to reduce nutrients, such as the use of chemical treatments to bind P in lakes (McDowell *et al.*, 2013). Presently there are multiple systems in place in New Zealand for reducing nutrient loads and preventing delivery of excess nutrients to coastal areas (Table 1.3, Journeaux *et al.*, 2017). For example, fencing to limit the access of livestock to water sources may reduce both the amount of excreta and urine being delivered directly into waterways, and the amount of physical soil disturbance from trampling hoofs (Journeaux *et al.*, 2017). However, the effectiveness of these measures at preventing runoff and nutrient loading can be limited, particularly in the case of fencing (Tanner *et al.*, 2003; Wilcock *et al.*, 2013).

Table 1.3: Methods of Nutrient Enrichment Mitigation.

	Method	Mitigation	Target	Description	Source/s
Reduction	Fencing (livestock control)	Point and diffuse source reduction	All	Limits the access of livestock to water sources, reducing the amount of excreta and urine being delivered directly into waterways, reduces the physical impact of large animals trampling ground, causing the loosening and loss of potentially nutrient enriched sediments into waterways. Fencing may be ineffective without riparian planting.	1
	Nutrient budgets	Legislation	N	Nitrogen caps are legal frameworks designed to limit the amount of nitrogen fertiliser applied to land. N caps limit intensive agriculture and ultimately can result in diversification of land use at a catchment scale	2
	Alum application	In situ capture and reduction	P	Whole lake treatment that doses the water body with aluminium sulphate which binds to P, before flocculating and sinking to form a sludge on the benthos. The sludge eventually will break down over time releasing the P back into the water column. The extent of possible ecological impacts are yet unknown.	3 & 4
	Bioremediation	In situ reduction, reuse & removal	N & P	The employment of living organisms, commonly micro-organisms, to collect and thereby remove pollutants from soil, water, or air. Plants, bacteria, mushrooms, micro- and macroalgae can be utilised in bioremediation of various substrates and waters.	5 & 6
Capture	Riparian planting and stabilisation	Diffuse capture	All	Reduces nutrient leaching from pasture. The plants effectively 'mop' up some of the excess nutrients, preventing runoff into waterways. The root systems of the plant can also stabilise the river banks reducing sediment loss and flood damage.	7 & 8
	Sediment traps	Point source capture	P	Traps that capture sediments containing P before they reach waterways. Have been effectively used to decrease levels of inorganic P.	9
	Wetlands	Diffuse & point source capture	N & P	Low lying areas planted with vegetation to capture and filter out sediments and nutrients from runoff. Can be highly effective nutrient sinks and filters using minimal space.	7
	Biochar	Point source capture	N & P	Biological charcoal made from organic waste products such as wheat husk or rice straw through a pyrolysis process. The resulting product has substantial biosorption ability and can remove various pollutants from industrial and agricultural wastewaters as well as soil. Biochar can be applied directly either into soil or wastewater and can be used as a filter media.	10 & 11
	Woodchip bioreactors	Point source capture and processing	N	Wood chip bioreactors are large pits built into pastures that contain course woodchips which are seeded with nitrifying bacteria. Designed to work with extensive draining systems to capture runoff, and urea within the pasture. Demonstrated to be effective as an in-situ treatment method for reducing nitrogen loads in farm runoff	12 & 13
	WWTPs	Point source processing	Primarily N	Waste Water Treatment Plants (WWTPs) are facilities utilised to treat and manage the release of municipal sewerage, household wastewater and industrial wastewaters	16, 17, 18
Disposal	Digestion ponds	Point source processing & disposal	Primarily N	Ponds that reduce the amount of N in point source discharges via denitrification and solids settlement. The settled solids form a sludge which is generally put to land disposal. After a period of both anaerobic and aerobic digestion the liquid waste is generally applied to pasture.	14
	Land disposal	Disposal	N & P	The disposal of nutrient enriched sludge from various sources such as WWTPs, farm digestion ponds, and land-based aquaculture. Management is critical to prevent underground seepage.	1 & 15

Sources: [1]Journeaux *et al.* (2017), [2]Barns and Young (2013), [3]Tempero (2015), [4]Vadas *et al.* (2007), [5]Allaby (2010), [6]Kidgell *et al.* (2014), [7]Tanner *et al.* (2003), [8]Wilcock *et al.* (2013), [9]Clarke *et al.* (2013), [10]Matovic (2011), [11]Yu *et al.* (2020), [12]Hudson *et al.* (2018), [13]Salimova *et al.* (2020), [14]Hickey *et al.* (1989), [15]Wiśniowska *et al.* (2019), [16]Zhang and Liu (2022), [17]Carey and Migliaccio (2009), [18]Holeton *et al.* (2011).

Agricultural waste is difficult to capture and mitigate due to its diffuse, non-point source nature (Houlbrooke *et al.*, 2010). Moreover, methods such as sediment traps and alum dosing do not remove the P from the environment and therefore it may still break down over time and become available once more (Vadas *et al.*, 2007). There is also evidence that a reduction in P does not equate to a parallel reduction in eutrophic symptoms such as HABs (Glibert, 2017). Major shifts in N:P ratios may cause further problems due to community shifts as species with differing optimal nutrient requirements gain competitive advantage in varied N:P ratios (Glibert, 2017). Therefore, even if P levels are reduced, an ecosystem may still be at risk of becoming or remaining eutrophic. Although the primary focus remains on N mitigation as this is commonly regarded as the predominant cause of eutrophication (Malone & Newton, 2020), successful mitigation methods will ideally reduce both N and P. Despite current management efforts, 24% of global anthropogenic nutrients released into coastal catchments are ultimately delivered into coastal waters such as estuaries (Malone & Newton, 2020). Moreover, there is potential for future quantities of nutrients arriving in coastal waters to increase due to legacy nutrients in groundwater discharged during previous decades (Burnett *et al.*, 2006). Therefore, there is a critical need to develop and implement in-situ nutrient mitigation technologies within estuaries to enable estuarine restoration and prevent further and/or future eutrophication (Duarte & Krause-Jensen, 2018; Yu & Gan, 2021). One such technology is algal bioremediation (Nielsen *et al.*, 2012; Neveux *et al.*, 2018; Bews *et al.*, 2021)

1.3 Algal Bioremediation

Algal bioremediation is the "use of live algae to remove excess nutrients or other contaminants from water" (Adey *et al.*, 2011; Sivakumar *et al.*, 2012; Lawton. *et al.*, 2013). Algal bioremediation is a growing industry that uses macro- or microalgae in both

fresh and marine waters (Nielsen *et al.*, 2012; Rose *et al.*, 2015; Neveux *et al.*, 2018; Bews *et al.*, 2021). Macroalgal bioremediation has been successfully implemented in a broad range of wastewater sources for nutrient mitigation, including aquaculture, agriculture, and other industries (Kebede-Westhead *et al.*, 2006; Godos *et al.*, 2009; Lawton. *et al.*, 2013). Studies have consistently shown that macroalgae can effectively and efficiently reduce nutrient concentrations in aquaculture wastewater, in particular concentrations of DIN (Roleda & Hurd, 2019). For example, cultivation of *Gracilaria vermiculophylla* in a pilot-scale Integrated Multi-Trophic Aquaculture (IMTA) experiment successfully removed 40.54 g m^{-2} $month^{-1}$ of nitrogen with a biomass productivity rate of 39.9 g (DW) m^{-2} day^{-1} in summer (Abreu *et al.*, 2011). Furthermore, studies conducted in Denmark, North America, and China, suggest large-scale macroalgae aquaculture can successfully extract significant quantities of nutrients from coastal waters (Fei, 2004; Kim *et al.*, 2014, 2015; Seghetta *et al.*, 2016; Saldarriaga-Hernandez *et al.*, 2020). For example, Chinese seaweed aquaculture produced 2 million tons dry weight of seaweed (mainly *Saccharina japonica* and *Gracilariopsis* spp.) in 2014, and the harvest of this seaweed removed approximately 75,000 t nitrogen and 9,500 t phosphorus from Chinese coastal waters (Zheng *et al.*, 2019). This corresponds to ca 5.6% of estimated total N-inputs and 40% of P-inputs, and results from a cultivation area of approximately 1,250 km^2 - a mere 0.3% of Chinese territorial waters (Xiao *et al.*, 2017; Hu *et al.*, 2021). Therefore, in-situ algal bioremediation could be used to reduce nutrient levels in estuarine habitats, restoring ecological balance through mitigation and prevention of eutrophication (Nielsen *et al.*, 2012; Neveux *et al.*, 2018; Bews *et al.*, 2021).

Macroalgae are primarily cultivated on a commercial scale for food products or commodities (Abreu *et al.*, 2011; Xiao *et al.*, 2017; Roleda & Hurd, 2019). The nutrient removal services the algae provide are generally considered a secondary ecosystem

service to the production of biomass (Gentry *et al.*, 2020); in-situ macroalgae cultivation for the primary purpose of bioremediation to reduce or prevent eutrophication is a relatively new concept that is gathering increasing interest (de Oliveira *et al.*, 2016; Nie *et al.*, 2020). However, algal bioremediation could be a cost-effective option for large-scale in-situ estuarine restoration (Adey *et al.*, 2013; Nwoba *et al.*, 2017). Developing in-situ mitigation strategies for nutrient enrichment are important in managing coastal eutrophication directly within estuarine basins (Kennish, 2002). In-situ algal bioremediation provides an alternative and complementary approach to the traditional land-based treatments described in section 1.2 and could facilitate estuarine restoration and prevent further and/or future eutrophication.

1.3.1 Algal Bioremediation & industry development in New Zealand

At present, macroalgal bioremediation is in the developmental stage in New Zealand, with a primary focus on freshwater (Craggs *et al.*, 2012; Mehrabadi *et al.*, 2015; Sutherland & Ralph, 2020; Stenton-Dozey *et al.*, 2021). Sustainable growth of the aquaculture industry in New Zealand is now a key focus of the New Zealand government, who have set an ambitious goal of NZ$3 billion in aquaculture sales by 2035 (N.Z.A.C., 2006; NZ Government, 2019). Much of this focus on aquaculture is on developing a macroalgal industry in New Zealand. Moreover, there has been increasing interest from other sectors (local government, private/commercial, and Iwi/Hapu) in developing macroalgal aquaculture in New Zealand; particularly in the marine space (N.Z.A.C., 2006; McGinnis & Collins, 2013; NZ Government, 2019). Primary industries in New Zealand have intensified production in recent decades to meet increasing export demands, causing significant ecological damage and public environmental concern (Bolan *et al.*,

2009). Therefore, New Zealand is in an ideal position to develop and implement algal bioremediation as a sustainable, and restorative technology.

1.4 Macroalgae ecology & physiology

1.4.1 Macroalgae ecology, & habitat tolerance

From rock-encrusting reds to giant forest-forming kelps, marine macroalgae or 'seaweeds' are ecologically important in many marine habitats (Wiencke & Bischof, 2012; Nelson, 2020). Seaweeds are found within the photic zone of coastal areas and provide a diverse range of services (Wiencke & Bischof, 2012; Nelson, 2020; O'Higgins *et al.*, 2020). These organisms are fundamental in nutrient cycling and habitat provision in coastal areas, and are broadly categorized into three taxonomic groups: Rhodophyta (red algae), Chlorophyta (green algae), and Ochrophyta (brown algae) (Wiencke & Bischof, 2012; Nelson, 2020). Macroalgae are found in habitats with a varied and dynamic range of often challenging conditions (Wiencke & Bischof, 2012; Nelson, 2020). Light is generally the most significant factor limiting the growth, survival, and distribution of macroalgae (Wiencke & Bischof, 2012; Roleda & Hurd, 2019). Moreover light can also cause macroalgae to alter its morphology; for example, it may grow longer blades/fronds, or alter pigmentation to better reach or absorb light (Dawes *et al.*, 1998; Orduña-Rojas *et al.*, 2002; Mata *et al.*, 2006; Charrier *et al.*, 2012; Wiencke & Bischof, 2012). Nonetheless, some macroalgae have adapted to light levels as low as 1 - 2% of surface PAR (Wiencke & Bischof, 2012; Nelson, 2020). Other species have adapted to particularly large fluxes in light availability through changes in photosynthetic efficiency as well as optimizing or inhibiting photosynthetic processes (Leukart & Lüning, 1994; Wiencke & Bischof, 2012; Roleda & Hurd, 2019). Nutrients in marine environments are often limited, particularly in warmer regions as the water column stratifies, and are the

second most limiting factor for macroalgal survival (Wiencke & Bischof, 2012; Roleda & Hurd, 2019). Therefore, many macroalgae have developed mechanisms to enable survival through nutrient 'droughts', including nitrogen stores and luxury uptake (Laycock & Craigie, 1977; Roleda & Hurd, 2019). Other challenges for macroalgae living in estuarine areas include water exchange through tidal cycles, freshwater influence, exposure/desiccation, and salinity fluctuation (Nelson, 2020; Thrush *et al.*, 2021). To combat these challenges many macroalgae species have unique cellular adaptations that enable their survival under extreme drying and/or irradiance, as well as broad temperature, and salinity shifts (Littler, 1980; Littler *et al.*, 1983; Carpenter, 1990; Wiencke & Bischof, 2012). The physiological characteristics that enable some seaweeds to cope with challenging conditions in estuarine habitats make these species particularly tolerant and robust (Kamer & Fong, 2000; Wiencke & Bischof, 2012; Karsten, 2012; Scherner *et al.*, 2013).

1.4.2 Nutrient & light requirements, assimilation & growth

Macroalgae require light and the essential macronutrients N, P and carbon (C) to grow (Roleda & Hurd, 2019). Nitrogen is generally considered most limiting; however, phosphorus may also be limiting in some cases (Roleda & Hurd, 2019). Macroalgae also require several micronutrients, however, these are not regarded as limiting in seawater (Wiencke & Bischof, 2012) and therefore are not discussed here. Macroalgae have three methods of nutrient uptake: passive diffusion, facilitated diffusion using channel proteins, and active transport where nutrients are transported into cells against concentration gradients (Roleda & Hurd, 2019). Passive diffusion is the least metabolically expensive uptake method (Roleda & Hurd, 2019). However, the ability to actively assimilate and store additional nutrients as luxury uptake either through facilitated diffusion or active

transport enables many species to cope with temporal variability in nutrient flux (Laycock & Craigie, 1977; Wiencke & Bischof, 2012; Roleda & Hurd, 2019).

1.4.2.1 Photosynthesis & The Light Spectrum

Macroalgal photosynthetic performance is dictated by chlorophyll content, accessory pigment type, and irradiance (Wiencke & Bischof, 2012; Roleda & Hurd, 2019). Accessory pigments give algae their colour, e.g., red algae gain their colour from phycobiliproteins, whilst browns have a dominance of the xanthophyll pigment fucoxanthin (Wiencke & Bischof, 2012). The different pigments and their arrangements within the cells of the alga are adapted to varying wavelengths of light and therefore increasing photosynthetic efficiency (Wiencke & Bischof, 2012). In deeper water, blue/green light is the most readily available as it penetrates deeper into the water column than other colours in the light spectrum (Figure 1.7; Wiencke & Bischof, 2012; Yarish *et al.*, 2012). As the phycobiliproteins of red alga principally absorb these spectra, red alga are often categorized as deep-water species (Wiencke & Bischof, 2012). However, this is frequently inaccurate as many species of red algae have other photosynthetic pigments and are commonly found in intertidal and shallow sub-tidal habitats. Moreover, thallus morphology and cell layers can alter the spectra of light the algae can absorb (Wiencke & Bischof, 2012). Photosynthetic performance and macroalgal growth are reliant on light quantity and quality, and are therefore directly impacted by irradiance loss (Leukart & Lüning, 1994; Wiencke & Bischof, 2012; Roleda & Hurd, 2019).

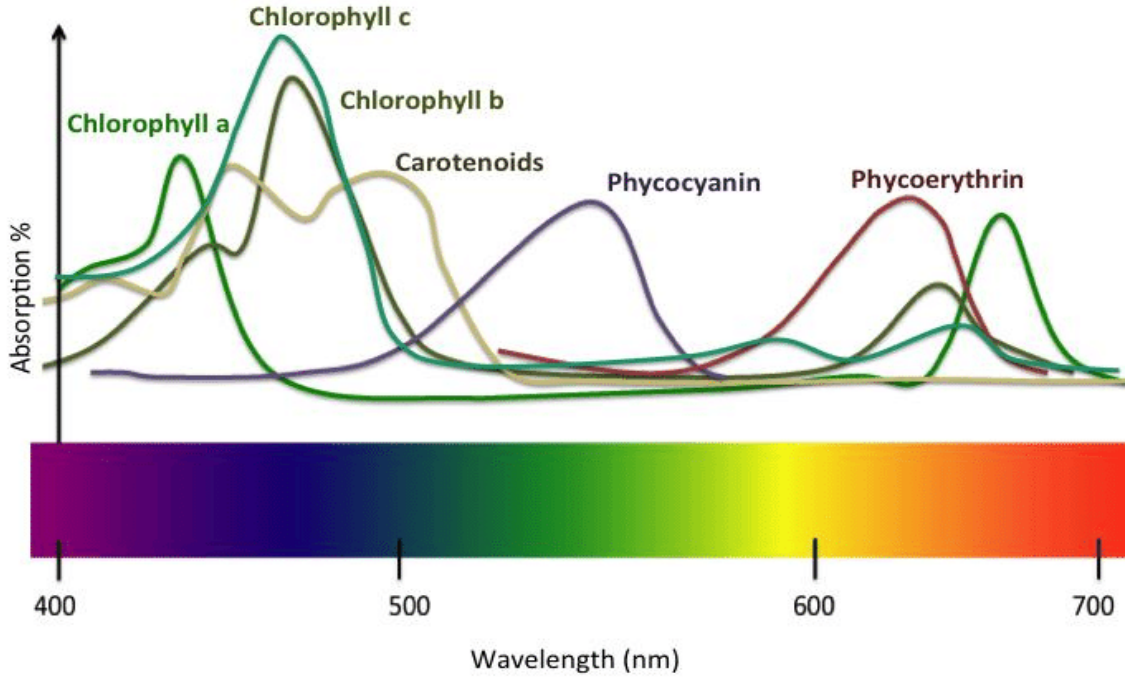

Figure 1.7: Spectra of light absorption for marine macroalgal pigments. Source: Yarish *et al.* (2012).

1.5 Effects of turbidity on macroalgae

Turbidity reduces optical clarity, reducing the amount of light or irradiance penetrating through the water column (Kitchener *et al.*, 2017; Gall *et al.*, 2019; Mangan *et al.*, 2020a). Turbidity is frequently caused by sedimentation, defined here as the "delivery and deposition of terrigenous sediments to marine environments" (Gall *et al.*, 2019; Booth, 2020; Mangan *et al.*, 2020a). As freshwaters transport these sediments, turbidity is often associated with cumulative impacts on coastal ecosystems due to the concurrent delivery of organic materials and nutrient-rich runoff in the freshwater and subsequent changes in salinity (Kennish, 2002; Green, 2006; Booth, 2020). Such cumulative effects can have extensive impacts on biodiversity, and ecological resilience, and may eventually lead to

shifts in dominant habitat type (as discussed above in section 1.1.1.) (Mangan *et al.*, 2020a; Thrush *et al.*, 2021).

1.5.1 Sediment delivery

New Zealand is a country with a comparatively high rate of sediment delivery from land to sea (Hicks *et al.*, 2011). Despite New Zealand being rated the 76th country in the world by size, covering just 0.2% of global landmass (Worldometer, n.d.), it is estimated to account for 1.7% of the total amount of sediment delivered to coastal regions worldwide (Hicks *et al.*, 2011). This relatively high rate of sedimentation is a result of several factors. New Zealand lacks many of the anthropogenic structures that aid in trapping and holding sediment, such as walls and dams which are frequently found in more developed regions with older infrastructure (Aubrey, 1993; Hicks *et al.*, 2011). Furthermore, extensive land-use modification has occurred in New Zealand over a relatively short period of time, resulting in large-scale shifts from native forest to clear pasture which has loosened the soil and increased sediment runoff (Table 1.4, Kennish, 2002; Hicks *et al.*, 2011; Plew *et al.*, 2020). Geography, high rainfall, and tectonic activity also contribute to high sedimentation rates in New Zealand's coastal space (Hicks *et al.*, 2011; Booth, 2020).

Table 1.4: Approximate sediment yields from catchments with differing land use in New Zealand. Source: Jones (2008)

Land use	**Estimated Sediment yield (tonnes/km^2/yr)**
Low hill country native catchment	10
Low hill country pasture catchment	100
Mountainous regions	1000
Mature urban catchments	100
Catchments with significant construction	1000

1.5.2 Turbidity and light limitations

Turbidity is a significant cause of light loss for algae attached on the benthos (Serôdio *et al.*, 2008). Suspended sediment particles causing turbidity can account for 80% of light variability and 56% of irradiance loss in estuaries around New Zealand (Gall *et al.*, 2019). Suspended sediments can vary in size, colour, and light absorption, all of which affect their optical properties and their contribution to coastal turbidity (Gall *et al.*, 2019). Fine terrigenous sediment is often made up of clay and silt particles (Wentworth, 1922; Green, 2006; Curran *et al.*, 2007). These are finer and more likely to be maintained in suspension, causing a greater loss of irradiance compared to larger sized particles that settle out of suspension more quickly (Wang, 1974; Gall *et al.*, 2019; Booth, 2020). The impacts of turbidity on macroalgae are dependent on the intensity and longevity of the turbid period (Gall *et al.*, 2019). However, long-term increases in turbidity combined with an increased storm frequency are likely to be the most detrimental (Lohrer *et al.*, 2004; Norkko *et al.*, 2006; Booth, 2020). Long-term loss of irradiance could cause reduced growth, nutrient assimilation, and photosynthetic performance of macroalgae in impacted estuarine environments (Gall *et al.*, 2019; Roleda & Hurd, 2019; Mangan *et al.*, 2020b; Blain *et al.*, 2021).

1.6 *Gracilaria transtasmanica* as a target species for algal bioremediation

1.6.1 Genus *Gracilaria*; morphology, physiology, and key characteristics

Red algae, or Rhodophyta, are the largest of the three macroalgae groups, and are represented by over 600 species in New Zealand (Nelson, 2020). Red algae vary widely

in colour, morphology, life history, and habitat preference (Wiencke & Bischof, 2012; Nelson, 2020). They are found in virtually all marine habitats down to depths with a minimum of 1% of surface irradiance (Wiencke & Bischof, 2012). However, only a limited number of species thrive in estuarine habitats, with members of the genus *Gracilaria* often found to be particularly prominent in sheltered harbours and estuaries (Wilcox *et al.*, 2001; Huanel *et al.*, 2020; Nelson, 2020). *Gracilaria* is a large genus of some 120 species found across both northern hemisphere and southern hemisphere temperate regions (Yarish *et al.*, 2012; Nelson, 2020). It is the second highest commercially valued genus of red macroalgae. Globally, 3.9 million tonnes of *G. chilensis* and *G. tenuistipitatum* are cultivated and harvested each year (Preuss *et al.*, 2020). Much of the interest in this genus is due to its diverse biomass applications. *Gracilaria* biomass is predominantly utilised in agar production, with harvests of this genus in 2015 contributing 91% of the biomass for industrial agar production (Mantri *et al.*, 2020). Various species within the genus have been proposed for, or are currently under development for, the globally growing aquaculture industry (Fei, 2004; Abreu *et al.*, 2011; Yarish *et al.*, 2012; Mantri *et al.*, 2020).

1.6.2 *Gracilaria transtasmanica* as a target species

Gracilaria transtasmanica is an indigenous red macroalga found in harbours and estuaries around New Zealand and Southern Australia (Figure 1.8, Wilcox *et al.*, 2007; Preuss *et al.*, 2020; Nelson *et al.*, 2021). The species is thought to have significant potential for domestication, bioremediation, aquaculture, and biomass applications (Preuss *et al.*, 2020; Nelson, 2020). *Gracilaria transtasmanica* has only recently been formally identified and was initially named as *Agarophyton transtasmanicum* (Preuss *et al.*, 2020), but later renamed *Gracilaria transtasmanica* (Gracilaria transtasmanica

G.M.Lyra, C.Iha, J.M.C.Nunes, C.C.Davis comb.nov.; Lyra *et al.*, 2021). *Gracilaria transtasmanica* appears to be a dominant intertidal estuarine species, tolerant to wide variation in salinity, exposure, desiccation, nutrient supply, and turbidity (Dudley *et al.*, 2022). This species has also been observed to contribute to and independently form nuisance blooms in nutrient-enriched areas (Nelson *et al.*, 2015; Dudley *et al.*, 2022). It is brown in colour and heavily branched in appearance and has a finer thallus and branchlets than the closely related *G. chilensis*. Specimens have been observed forming dense mats of biomass with strands up to 40cm long on the tidal flats in apparent optimum conditions (B. G. Ross, unpublished data, Figure 1.8). In New Zealand, the species has been recorded in the Auckland, Bay of Plenty, Canterbury, Southland, and Otago regions and is considered likely to occur throughout New Zealand estuaries and harbours (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Nelson, 2020; Huanel *et al.*, 2020; Preuss *et al.*, 2020; Dudley *et al.*, 2022).

Gracilaria transtasmanica is consistently found in the upper reaches of highly tidal estuaries (B. G. Ross, unpublished data), often in regions with high freshwater influence, intense land use, and agricultural runoff (Scholes, 2015; Orchard, 2016). The species also co-exists with *G. chilensis,* with no apparent competition (Huanel *et al.*, 2020). However observations in the Maketu Estuary in the Bay of Plenty suggest they may inhabit differing intertidal habitats, with *G. transtasmanica* often fully emerged on upper tidal levels and, whilst *G. chilensis* is generally submerged in mid to lower intertidal channels (B. G. Ross, unpublished data). *Gracilaria transtasmanica* appears well suited to New Zealand estuaries and tolerant to a broad range of dynamic conditions (Nelson, 2020; Dudley *et al.*, 2022).

Figure 1.8: Clockwise - *G. transtasmanica* forming thick mats on emerged mudflats, cultured biomass, and healthy individual specimens showing differing morphologies.

Gracilaria transtasmanica produces a nitrogen-rich amino acid called gigartinine (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Huanel *et al.*, 2020; Preuss *et al.*, 2020; Dudley *et al.*, 2022). Gigartinine is a free amino acid [5-(3-amidinoureido)-2-aminovaleric acid] (Figure 1.9) found in many red seaweeds and is commonly considered to serve as a nitrogen store (Laycock & Craigie, 1977; Preuss *et al.*, 2020; Huanel *et al.*, 2020). Gigartinine levels can be seasonally variable and have been found to comprise from 9 to 35% of extractible N in *Gracilaria flabelliformis* biomass (Laycock & Craigie, 1977). The gigartinine molecule has a Carbon to Nitrogen ratio (C:N) of 5:7 (Laycock & Craigie, 1977; PubChem, 2005), compared to aspartic acid (one of the most abundant amino acids in plants and animals) which has a ratio of 2:4 (PubChem, 2004). Previous work has shown that *G. transtasmanica* can rapidly produce and accumulate gigartinine in response to increased nitrogen availability (Wilcox *et al.*, 2007), suggesting that it may have rapid nutrient uptake rates. Therefore, *G. transtasmanica* has potential as a novel target species for the bioremediation of nutrient-enriched estuarine habitats (Preuss *et al.*, 2020). The presence of a nitrogen store could also provide the alga with an advantage when

temperature and light conditions become optimum in spring/summer but nutrient concentrations in the water column may be low (Laycock & Craigie, 1977; Huanel *et al.*, 2020). Thus, the presence of gigartinine may enhance this species' robustness and tolerance to estuarine conditions.

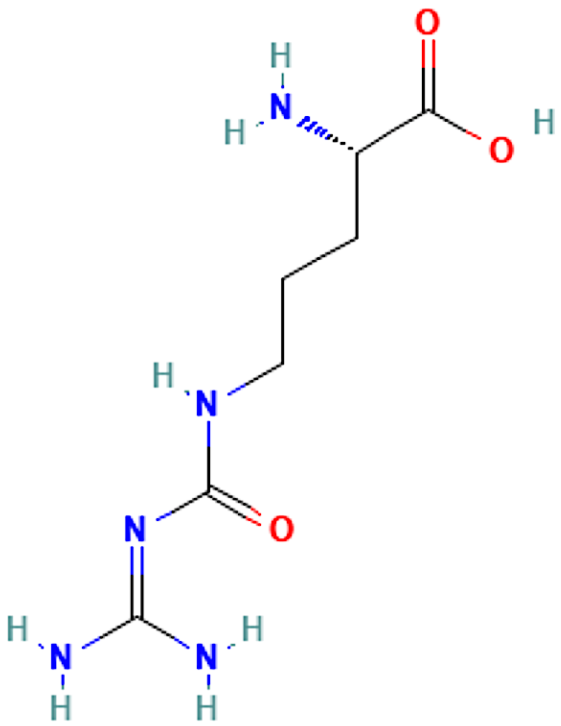

Figure 1.9: The chemical structure of the amino acid Gigartinine. Sourced from: PubChem (2005).

Domestication of a novel aquaculture species can be a lengthy and complicated process requiring a considerable amount of research (Liao & Huang, 2000). Consequently, novel target species that are related to current commercial species are generally considered preferable as existing research and cultivation protocols for current species can be used to fast-track domestication and enable successful cultivation of the new target species (Rose *et al.*, 2015). *Gracilaria tikvahiae*, *G. chilensis,* and *G. vermiculophylla* are commercially cultured species of morphological and genetic similarity to *G. transtasmanica* with an international aquaculture industry of considerable size and value (Abreu *et al.*, 2011; Mantri *et al.*, 2020; Preuss *et al.*, 2020). As a result, it should be

relatively straight-forward to establish aquaculture techniques for *G. transtasmanica* based on approaches currently utilised for these related species (Kain & Destombe, 1995; Yarish *et al.*, 2012). The bioremediation potential of *G. transtasmanica* coupled with the possibility of a fast-tracked domestication process and its apparent robustness to dynamic estuarine conditions make *G. transtasmanica* an ideal target for in-situ bioremediation in estuaries.

1.6.2.1 Biomass uses and value-adding products

Macroalgal biomass uses and products are diverse (de Almeida *et al.*, 2011; Sudhakar *et al.*, 2019). Cosmetics, agar plates, fertilisers, bio-stimulants, aquaculture feeds, biochar, nutraceutical supplements, and biofuels, are all products that contain macroalgal biomass or extracts (de Almeida *et al.*, 2011; Sudhakar *et al.*, 2019). *Gracilaria transtasmanica* could potentially contribute to the agar industry, as this genus is known to produce the cell-wall polysaccharide agar (Tello-Ireland *et al.*, 2011). In fact, almost all commercial agar is sourced from the genus *Gracilaria*, underpinning its value as an aquaculture species (Yarish *et al.*, 2012; Mantri *et al.*, 2020). Additionally, this species has similar characteristics to other *gracillariids* used in aquaculture feeds for species such as Paua (New Zealand abalone) (Naidoo *et al.*, 2006; Cian *et al.*, 2019). Furthermore, as *G. transtasmanica* can accumulate nitrogen rich gigartinine, there is potential for biomass to be used in fertilisers and bio stimulants (Aitken & Senn, 1965; Salcedo *et al.*, 2020). Additionally, high concentrations of N in the biomass may lend this species to use in dietary supplements as a high N content is generally indicative of a high protein content (Taboada *et al.*, 2013; Kavale *et al.*, 2017). In combination, these characteristics suggest *G. transtasmanica* biomass has a broad range of possible applications and biproducts.

This species is therefore likely to have commercial value in addition to bioremediation and restorative values.

1.7 Thesis aims and intentions

The primary aim of this thesis is to investigate the potential of *G. transtasmanica* as a novel target species for the bioremediation of nutrient enriched New Zealand estuaries. Specifically, this thesis aims to isolate and culture *G. transtasmanica*, and assess the nutrient removal ability and environmental tolerance of this species. Chapter two will assess the seasonal effects of turbidity on the photosynthetic performance, biomass productivity, nutrient removal, and biomass quality of *G. transtasmanica*. This chapter will demonstrate the species' ability to assimilate nutrients under conditions representative of New Zealand estuaries. Chapter three will investigate the tolerance of *G. transtasmanica* to abiotic estuarine conditions such as low salinity, irradiance loss, and desiccation exposure. This chapter will provide an indication of the environmental conditions that *G. transtasmanica* can be cultivated under and the likely effects of variations in these conditions on growth. Finally, Chapter four will provide a general discussion and synthesis of the experiments carried out in the previous two chapters, including relevant recommendations for future experimental work and development with *G. transtasmanica.*

Chapter 2
Nutrient assimilation and biomass productivity of *Gracilaria transtasmanica* in nutrient-enriched & turbid conditions

2.1 Introduction

Estuaries worldwide are becoming increasingly nutrient-enriched, causing widespread eutrophication (Plew *et al.*, 2018a; Brown, 2019; Malone & Newton, 2020). Nutrients, in particular inorganic nitrogen, are now being delivered to coastal waters at an unprecedented rate largely due to increasing waste from a growing population and an intensifying agricultural sector (Howarth, 2008; Journeaux *et al.*, 2017; Stewart *et al.*, 2018; Malone & Newton, 2020; Plew *et al.*, 2020). Once in coastal waters, excess nutrients can nourish algal blooms, leading to hypoxia, eutrophication, general habitat degradation, and ultimately a loss of ecosystem services (Ellis *et al.*, 2000; Bricker *et al.*, 2008; O'Higgins *et al.*, 2020). Reducing excess nutrients in coastal waters is therefore critical to preserve and sustain estuarine ecosystem functionality (Bricker *et al.*, 2008; Rose *et al.*, 2015). Mitigation efforts to date have largely focused on land-based capture and/or reduction of excess nutrients in attempts to reduce nutrient delivery to coastal waters via freshwater-ways and submarine groundwater leaching (Tanner *et al.*, 2003; Clarke *et al.*, 2013; Rose *et al.*, 2015; Journeaux *et al.*, 2017; Stewart *et al.*, 2018). However, despite these nutrient capture and mitigation techniques, 24% of global anthropogenically sourced nutrients released in coastal catchments reach coastal waters (Malone & Newton, 2020). At present, there is no viable in-situ method for reducing elevated nutrient levels within at-risk coastal waters such as estuaries.

In-situ macroalgal bioremediation is a potential solution for reducing nutrients in enriched coastal waters such as estuaries (Fei, 2004; Kim *et al.*, 2014; Rose *et al.*, 2015; Kim *et al.*, 2015; Seghetta *et al.*, 2016). In-situ macroalgal bioremediation is the cultivation of live macroalgae directly in nutrient-enriched waters for the primary purpose of nutrient assimilation (Lawton. *et al.*, 2013; Kim *et al.*, 2014, 2015; Seghetta *et al.*, 2016). Application of large-scale macroalgal cultivation for bioremediation in coastal waters can successfully reduce concentrations of dissolved nutrients (Fei, 2004; Kim *et al.*, 2014, 2015; Seghetta *et al.*, 2016; Saldarriaga-Hernandez *et al.*, 2020). For example, based on recorded productivities and contents of nitrogen and phosphorus in the biomass, cultivation of *Saccharina latissma* and *Gracilaria tikvahiae* in the Bronx River estuary has the potential to remove up to 430 kg nitrogen ha^{-1} yr^{-1} (Rose *et al.*, 2015). Similarly, in China's severely eutrophic Hangzhou Bay (China's largest bay), cultivation of *Gracilaria verrucose* in a 1.72 km^2 area reduced the concentration of ammonium (NH_4-), nitrate (NO_3-), and phosphate (PO_4-) by 54%, 76%, and 49% respectively in a single year (Huo *et al.*, 2011).

Estuaries are highly dynamic and challenging environments (Green, 2006; Statham, 2012). Therefore, target macroalgae species for in-situ bioremediation must be robust (Lawton. *et al.*, 2013; Kim *et al.*, 2014). In particular, estuaries have high rates of sedimentation causing turbidity and a subsequent reduction in the light available for photosynthesis (Serôdio *et al.*, 2008; Gall *et al.*, 2019; Mangan *et al.*, 2020b; Blain *et al.*, 2021). Reduced photosynthetic function causes lower rates of productivity and can therefore reduce the bioremediation potential of a given macroalgae species (Serôdio *et al.*, 2008; Lawton. *et al.*, 2013; Rose *et al.*, 2015; Mangan *et al.*, 2020b; Blain *et al.*, 2021). As a result, target species must be able to maintain reasonable growth rates under turbid and light limited conditions. Additionally, target

species must be effective at assimilating nutrients, particularly nitrogen (N), and ideally have high biomass productivity, quality, and value (Lawton. *et al.*, 2013; Rose *et al.*, 2015).

Several species of *Gracilaria* are cultured globally and are generally robust, relatively fast-growing seaweeds with existing commercial applications (Fei, 2004; Kim *et al.*, 2014). Moreover, species of *Gracilaria* have been successfully used for bioremediation of estuarine habitats in North America and China (Fei, 2004; Kim *et al.*, 2014; Rose *et al.*, 2015; Kim *et al.*, 2015). *Gracilaria transtasmanica* is a red macroalga, native to New Zealand, thought to hold potential for cultivation, particularly for in-situ estuarine bioremediation (Preuss *et al.*, 2020). *Gracilaria transtasmanica* is found in intertidal habitats in harbours and estuaries around New Zealand and Southern Australia and can form nuisance blooms in some nutrient enriched estuaries (Nelson *et al.*, 2015). It appears to be tolerant to wide variations in salinity, exposure, nutrient flux, temperature, and turbidity (Preuss *et al.*, 2020; Nelson, 2020). Moreover, unlike the sympatric and morphologically similar *Gracilaria chilensis, G. transtasmanica* produces a unique amino acid known as gigartinine, which is only found in select red algae and is thought to act as a nitrogen reservoir (Laycock & Craigie, 1977; Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Preuss *et al.*, 2020). *Gracilaria transtasmanica* can rapidly accumulate gigartinine in response to increased nitrogen availability, such as when it is grown in nutrient-rich water (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Preuss *et al.*, 2020). Therefore, *G. transtasmanica* may assimilate and store nitrogen at a higher rate than comparable species without the presence of gigartinine (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Preuss *et al.*, 2020). Furthermore, established cultivation methodologies exist for other species of *Gracilaria* (Rose *et al.*, 2015), creating the potential to fast-track domestication of *G. transtasmanica*. For these reasons, *G. transtasmanica* has potential as a novel target species for in-situ bioremediation of nutrient-enriched estuarine habitats.

The primary aim of this study is to investigate the suitability of *G. transtasmanica* for in-situ bioremediation of nutrient-enriched and turbid New Zealand estuaries. The secondary aim is to assess seasonal shifts in the growth and nutrient assimilation ability of *G. transtasmanica*. To achieve these aims, *G. transtasmanica* biomass will be domesticated from natural populations to establish stock cultures. Biomass productivity and nutrient removal rates of *G. transtasmanica* will then be assessed under varied nutrient and sediment treatments. Experiments will be conducted in outdoor cultures under ambient summer and winter conditions to provide an assessment of performance under conditions representative of those that would be experienced in natural estuarine habitats. These experiments will enable a thorough assessment of the effects of turbidity on the photosynthetic performance, productivity, and nutrient assimilation ability of *G. transtasmanica* and will indicate whether this species can be used for in-situ bioremediation of estuaries throughout the year.

2.2 Methods

2.2.1 Sample collection & identification

Samples of *Gracilaria* were collected from intertidal habitats at three sites each in the Maketu and Little Waihi estuaries in the Bay of Plenty region of New Zealand in February 2021 (Appendix 2.1). Most samples were growing attached to the benthos in silty/sandy areas of the upper-intertidal zone of the estuaries, forming large, and at times thick mats of biomass; a few samples were found unattached. Samples were transported in seawater taken from the collection sites to the University of Waikato Coastal Marine Field Station, Tauranga, New Zealand. Upon return to the laboratory, each sample was placed into a separate plastic bucket filled with filtered nutrient-enriched (Varicon Aqua Cell-Hi F2P, 12.3 mg N L^{-1}, and 1.1 mg P

L^{-1}) seawater and maintained in an environmentally controlled room (18ºC, 12:12 light:dark cycle, at 200 µmol photons m^{-2} s^{-1} using cool fluorescent lights). Buckets were provided with gentle aeration by a continuous stream of filtered air entering through multiple inlets around the base of the buckets.

Assessments of morphological characteristics suggested several samples were likely to be *G. transtasmanica.* However, this species often co-exists with *G. chilensis*, and it can be difficult to differentiate between these species based only on morphology (Huanel *et al.*, 2020). Therefore, species identification was determined using a recently developed genetic tool that discriminates between the two species using the cytochrome c oxidase subunit I gene (5P-COI) and the primers Agarophyton_COI_F, Agarophyton_COI_R, Agarophyton_chilense_COI_F, and Agarophyton_transtasmanicum_COI_F (Huanel *et al.*, 2020). Approximately 100 mg of fresh tissue from each sample was homogenized using a Precellys Evolution Homogenizer (Bertin Instruments, France) and genomic DNA was extracted using a DNeasy® Plant Pro Kit (Qiagen, Germany) following the manufacturer's instructions. DNA was amplified in 25 µL PCR reactions containing 12.5 µL MyTag HS Red Mix (MilliporeSigma, Germany), 0.4 µM of each primer, and 1 µL of genomic DNA. PCRs were run on an Applied Biosystems SimplyAmp Thermal Cycler (Thermo Fisher Scientific, USA) and comprised of an initial denaturation phase at 95 °C for 1 min, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Five µL of PCR product was run on a 2% agarose gel and amplicon size was determined using a size standard marker (Bioline HyperLadder™ 25 bp). Samples were identified as *G. chilensis* or *G. transtasmanica* based on the presence of bands in the expected size range on the gel.

All samples confirmed as *G. transtasmanica* were combined into a single master stock culture. The biomass then was grown in free-floating (tumble) cultures under the same conditions described above for three months prior to the start of the first experiment to ensure acclimation to culture conditions and allow sufficient time for biomass to upscale. Stock cultures were then continuously maintained for the next six months and used to supply biomass for both the summer and winter experiments.

2.2.2 Sediment collection & processing

A single bulk sample of sediment (approximately 2.5 kg dry weight (DW)) was collected from the Kaituna Cut area of the Maketu Estuary in the same location where *G. transtasmanica* samples were collected. The sediment was transported in buckets to the University of Waikato Coastal Marine Field Station where it was dried in an oven at 60 °C for 48 - 72 hours. Sediment particles smaller than 125 µm were isolated by filtering the dried sediment through a 125 µm sieve plate using an Endecott's EFL 2000 Test Sieve Shaker. All particles larger than 125 µm were discarded as sediments under 125µm consist of very fine sands down to silt and clay particles (Wentworth, 1922) and are representative of the high percent of clay sediments commonly found both deposited and suspended in estuaries with high terrigenous sediment loads (Green, 2006; Curran *et al.*, 2007). Three random subsamples of the filtered sediment were analyzed using a laser diffraction particle size analyzer (Mastersizer 3000) to assess size class and distribution. The sediments showed a size distribution from fine clay to fine sand, with a mean particle size of 34 µm, classifying the samples as coarse silt on the Udden-Wentworth scale (Appendix 2.2). N and P content in the sediment was analyzed by Hill Laboratories following standard methodology. The sediment had an N content of 500mg/100g

DW, and a P content of 0.004mg/100g DW. The sediment was stored in airtight plastic containers with silica sachets and was used in both the summer and winter experiments.

2.2.3 Assessing biomass productivity and bioremediation performance

To determine the bioremediation potential of *G. transtasmanica* and the effect of turbidity on bioremediation performance, a full factorial experiment with 3 sediment loading rates and 2 nitrogen concentrations was conducted. Sediment loading rates of 0 g L^{-1}, 0.02 g L^{-1}, & 0.25 g L^{-1} were tested. The 0.02 g L^{-1} loading rate represents background turbidity and is based on the mean total suspended solid loads recorded in local estuaries (Scholes, 2015). The 0.25 g L^{-1} loading rate represents turbid conditions and is based the amount of sediment that was required to generate an approximate 40 % loss of irradiance in culture buckets, as is typically found in relatively turbid estuaries (Gall *et al.*, 2019; Babuder, 2021). Nitrogen concentrations of 1 mg L^{-1} and 10 mg L^{-1} dissolved inorganic nitrogen (DIN) were tested. The 1 mg L^{-1} treatment represents background concentrations and is based on regional estuary water quality monitoring data showing the mean DIN concentration in local estuaries is 0.3 mg L^{-1} (Scholes, 2015). As the cultures were maintained in batch mode (e.g., not flow through) and nutrients were provided once every 4 days, this DIN concentration of 0.3 mg/L was taken as an approximate daily rate and was equated to a rate of 1 mg L^{-1} over a 4-day batch cycle. The 10 mg L^{-1} treatment was selected to represent nitrogen enrichment. The experiment was conducted in winter (July 2021) and then repeated in summer (January 2022) to determine any seasonal differences in performance. Identical procedures and methods were used for both experiments.

Prior to the start of each experiment, biomass from stock cultures was transferred into the Facility for Aquaculture Research of Macroalgae (FARM), University of Waikato, Tauranga,

New Zealand – an outdoor recirculating aquaculture system within a greenhouse. Biomass was maintained in 20 L plastic buckets in nutrient-enriched (Varicon Aqua Cell-Hi F2P, 12.3 mg N L^{-1}, and 1.1 mg P L^{-1}) filtered seawater with a stocking density of 2 - 3 g L^{-1} for seven days before the start of each experiment to enable acclimation to outdoor conditions. Each experiment was immediately started after the seven-day acclimation period. Five replicate cultures of each treatment combination were grown in batch culture in 5 L plastic buckets in the FARM under ambient natural light (total of 30 replicates). Buckets were placed in a temperature-controlled water bath to minimize large temperature fluctuations in the small bucket volume. Replicate buckets were filled with nutrient-enriched filtered seawater from two 63 L bulk stock solutions. These stock solutions were made immediately before the start of the experiment and before each harvest using filtered seawater. Nitrogen was added to the seawater in the form of nitrates (30405.9 mg L^{-1} $NaNO_{3-}$) to achieve concentrations of 1 and 10 mg L^{-1}, and phosphorus was added in the form of phosphates (8924.9 mg L^{-1} $H_2NaPO_4.2H_2O$) to achieve an N:P ratio of 10:1. A single grab sample was taken from each stock solution at the beginning of the experiment and on each harvest day and was frozen for later analysis. Throughout the experiments, the water temperature of the cultures was logged every 15 minutes using HOBO pendant water temperature loggers (MX2201, Onset) placed inside one culture bucket in each block (see below). Photosynthetically active light was logged every five minutes using a full spectrum quantum sensor logger (SQ-500-SS, Apogee) placed inside the greenhouse. Additionally, the irradiance in each replicate bucket was measured manually each day between 11 am and 1 pm, using an LI-1500 underwater (UWQ9463) digital light logger. The average water temperature of the cultures was 18.1 °C $\pm$ 0.2 S.E. in the winter experiment and 21.7 °C $\pm$ 0.5 S.E. in the summer experiment (Fig 2.1). Cultures received a mean photosynthetically active radiation of 260 µmol photons m^{-2} sec^{-1} $\pm$ 5.4 S.E. in the winter experiment and 867 µmol photons m^{-2} sec^{-1} $\pm$ 3.2 S.E. in the summer experiment (Figure 2.1).

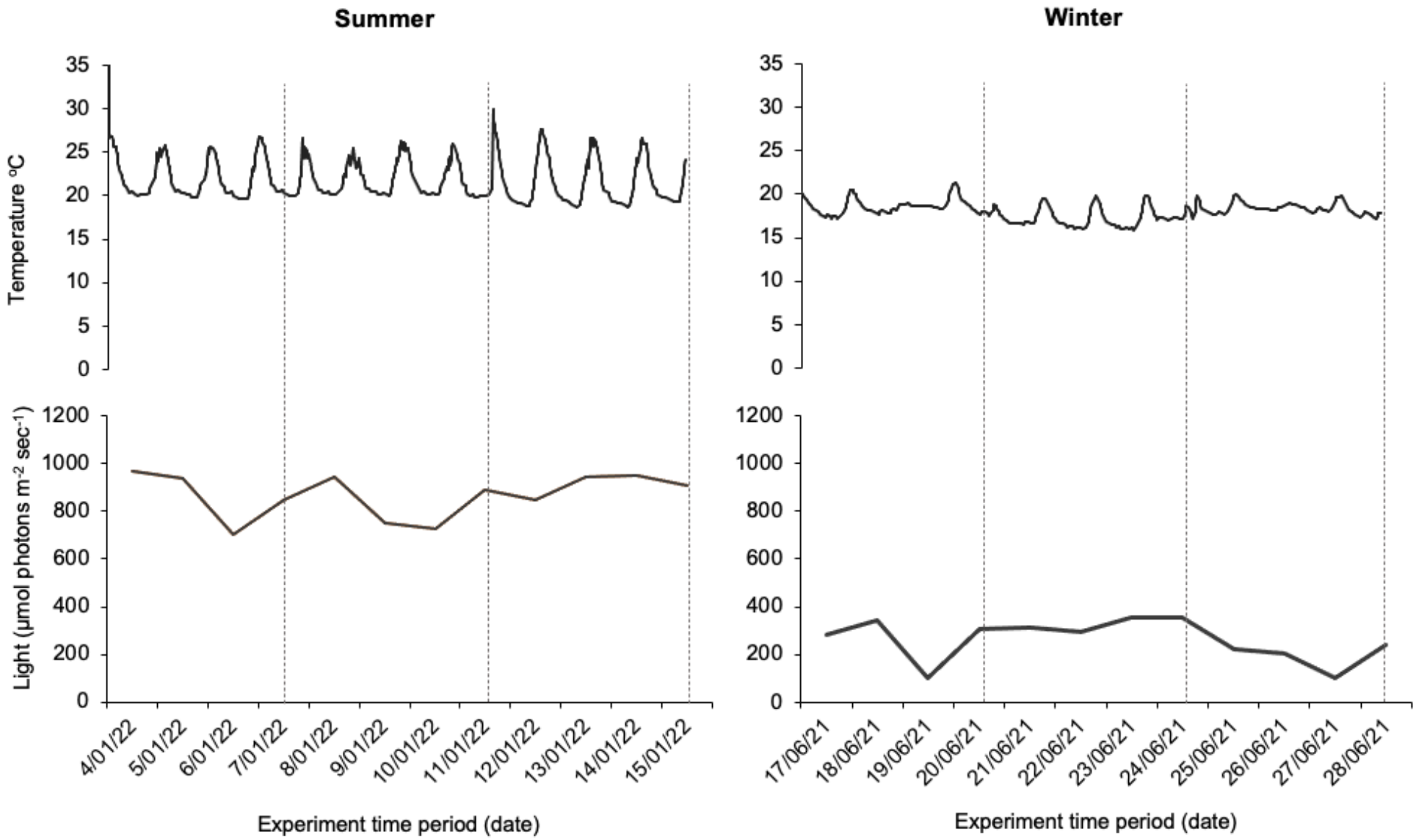

Figure 2.1: Water temperature in experimental buckets (top panel) and irradiance in the FARM (bottom panel) during the summer (left) and winter (right) experiments. Vertical dashed lines show harvest times on the 4th, 8th, and 12th days of the experiments

To maintain sediment constantly in suspension during the experiment, each replicate bucket was supplied with a continuous stream of air through six ceramic cylindrical air stones fitted to inlets around the base of the bucket. To represent natural settings in which *G. transtasmanica* grows primarily attached to the benthos, the biomass was placed in plastic mesh envelopes with a 3oz lead sinker attached to the underside to keep the mesh submerged on the bottom of the bucket. A large mesh size (1.5 cm^2) was used to minimize any shading effects. Replicate buckets were placed in a standard block design with five blocks, each containing one replicate bucket of each treatment combination. The position of buckets in each block was randomized using a random generator sequence produced using the 'Sample' function in *r Studio* (version 2021.09.1). The position of buckets within each block was rotated daily by shifting each bucket one position in a clockwise direction. Randomization was reset on each harvest day.

Buckets were harvested after four days, one block at a time. The biomass from each replicate was removed from the mesh envelope and placed in an individual cloth mesh bag. This mesh bag was course enough to allow fine sediment to filter through the cloth. The algae and bag were rinsed in filtered seawater to remove any sediment that had settled on the algae, and the harvest bag and algae were then spun in a centrifugal spin dryer (Spindel dryer SPL65) for 3 minutes. Biomass was removed from the harvest bags, weighed to record fresh weight (FW) and then 8 g of the harvested biomass was placed back in the mesh envelope and returned to the replicate bucket to reset stocking density. Any remaining biomass was retained for analysis (Section 2.2.5). The water in each replicate bucket was replaced with fresh stock solution at each harvest. This harvest and reset process was repeated a further two times, providing for a total of three consecutive harvests over twelve days. On the final day of the experiment (harvest three), these procedures were repeated exactly except the entire biomass was retained for analysis (Section 2.2.5).

2.2.4 Chlorophyll a fluorescence

Chlorophyll fluorescence was recorded as a measure of cell health and resilience under challenging conditions (Stirbet & Govindjee, 2011; Figueroa *et al.*, 2013) using a pulse amplitude-modulated (PAM) fluorometer (Junior-PAM Walz, Effeltrich, Germany). Changes in chlorophyll fluorescence yield caused by processes affecting photosynthetic activity and performance can be observed through PAM measurements (Serôdio *et al.*, 2008; Stirbet & Govindjee, 2011; Figueroa *et al.*, 2013). These measurements enable the assessment of Photosystem two (PSII) and are important in understanding photo-inhibitory damage that may occur due to exposure to challenging conditions (Mata *et al.*, 2006; Serôdio *et al.*, 2008; Townsend *et al.*, 2018). Effective photochemical quantum yield (Y(II)) is measured by the PAM fluorescence excited by consistently timed light pulses (Figueroa *et al.*, 2013). Changes in the signal returned through these light pulses indicate shifts in chlorophyll fluorescence and therefore changes in photochemistry in PSII (Figueroa *et al.*, 2013; Figueroa *et al.*, 2013). The functioning and potential long-term damage of PSII and therefore stress resilience under challenging conditions can be indicated by changes in optimal quantum yield, calculated with measurements of minimum and maximum yield (F_v/F_m). This is measured by using dark-adapted samples that experience a fluorescence induction known as the Kautsky effect when exposed to light, by placing samples in the dark before measuring F_v/F_m (Schreiber *et al.*, 1995; Stirbet & Govindjee, 2011). Low and high-level light pulses are used to measure F_v/F_m to indicate the physiological state of the photosynthetic cells (Stirbet & Govindjee, 2011; Figueroa *et al.*, 2013). Higher levels of effective (Y(II)) and optimal quantum yield (F_v/F_m) show healthy photosynthetic cells and processes, while lower effective quantum yields show the biomass is under stress causing short term damage often linked with high irradiance levels (Häder & Figueroa, 1997; Mata *et al.*, 2006). The biomass can recover from short term damage

if the stressor is reduced or removed (Häder & Figueroa, 1997; Häder *et al.*, 1998; Mata *et al.*, 2006). However, low levels of optimal quantum yield (F_v/F_m) show long-term damage to the cells which the biomass may recover from over a slower time scale but may also be unable to repair at all (Häder & Figueroa, 1997). PAM measurements, both Y(II) and F_v/F_m, were taken for each replicate culture on the experiment setup day, and for each of the three harvests. Y(II) was measured immediately after the biomass had been harvested and weighed; following this, a subsample of the biomass from each replicate was then placed in full darkness for 10 minutes, after which F_v/F_m was measured.

2.2.5 Biomass analyses

Excess harvested biomass not restocked back into cultures was placed into pre-weighed falcon tubes and dried on-site in a freeze dryer (*BUCHI LyovaporTM* L-200 Freeze Dryer) for approximately 72 hours, and then reweighed to provide an individual FW:DW ratio for each replicate culture, for each harvest. Samples of biomass from each replicate from each harvest were analyzed for ash content (combustion in the air at 600 °C for 5h). Samples of biomass from the initial biomass that was used to stock experimental replicates and from the final harvest of each replicate were also analysed for nitrogen, sulfur, carbon, and hydrogen by combustion in pure oxygen in an elemental analyser followed by separation and quantification by gas chromatography with a thermal conductivity detector (GC-TCD, Glasson *et al.*, 2017). All analyses were conducted commercially by OEA Labs (http://www.oealabs.com, Callington, UK). Ash-free dry weight (AFDW) productivity was calculated using the formula, $P = \{[(Bf - Bi)/FW{:}DW]*(1\text{-}ash)\}/ A/T$, where Bf and Bi are the final and initial algal biomasses (g FW), FW:DW is the fresh weight to dry weight ratio, ash is the proportional ash content of the dried biomass, A is the area (m^2) of culture tanks and T is the number of days in

culture. Total N removed was calculated for each replicate for both the winter and summer experiments by multiplying the biomass growth (mg DW) in the final harvest by the N content (% DW) this provided the total mg N removed in each replicate. N removal rate (mg N removed per g DW biomass growth) was then calculated by dividing the total N removed by the biomass growth (mg DW).

2.2.5.1 FAAs: Gigartinine

Free amino acids (FAAs) were measured in samples of biomass from the final harvest of each replicate and from the initial biomass that was used to stock experimental replicates from both winter and summer experiments using the Ninhydrin reaction method described by Friedman (2004). This method is highly reproducible and provides a relative measure of FAAs between samples and treatments (Starcher, 2001; Zhu *et al.*, 2009; Contreras-Martos *et al.*, 2018). The dried biomass samples were milled prior to water (RO) extraction (50 mg/5 mL), at 40°C for 24 hours with stirring. The extracts (1 mL) were then clarified by centrifugation (13,000 rpm for 5 minutes) and diluted by 1 in 20. To derivatise FAAs, 250 µL of sample or standard and 250 µL of Ninhydrin reagent (Starcher, 2001) was added to a 2 mL HPLC tube with 3 mm stirrer bar and heated at 100°C with stirring for 10 minutes. Ninhydrin derivatised samples and standards (200 µL) were then pipetted into a 96-microwell plate and the absorption of the generated Ruhemans purple measured at 575 nm using a BMG nanoStar plate reader. Due to its structural similarities with gigartinine, arginine was used to calibrate the assay (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007). FAA content was calculated as equivalents of the known concentrations of arginine (concentration range 5 – 40 µL mL^{-1}) in the calibration curve created from the standards. Some samples were either too high or low in FAAs to provide an accurate absorption measurement and were consequently diluted as appropriate (e.g., 1 in 10, or 1 in

40). Quality control was monitored using two technical replicates for each sample and analyses were repeated for samples with standard deviation greater than 10% of the mean.

2.2.6 Water quality

Changes in nutrient concentrations in the culture water were measured in the final 4-day growth period of the winter experiment to provide a further estimate of nutrient removal. In addition to the experimental replicates containing algae, five replicate buckets of each sediment and nutrient treatment combination were set up. These controls were identical to experimental replicates except that they were not stocked with any algae. Water samples were taken from the nutrient stock solutions that cultures were restocked into at the start of the final 4-day growth period, and from each replicate bucket (algae and control replicates) immediately before the final harvest. Water samples were analysed for ammonia, nitrate, nitrite (summed to calculate DIN), and DRP. All analyses were conducted commercially by Hill Laboratories following standard methodology. Differences in the concentration of DIN and DRP in the water sample taken from the starting stock solutions and the water samples taken from each replicate algal culture at the end of the 4-day growth period were calculated. The average decrease in DIN and DRP in the corresponding control treatments over the 4-day experimental period was then subtracted from this difference to account for any non-algae-related loss of DIN or DRP within treatments. The resultant value was used to provide a measure of bioremediation performance during winter conditions in addition to that calculated using biomass N content.

2.2.7 Statistical analyses

All statistical results are presented as the mean ± S.E. Factorial ANOVAs were used to test for differences in AFDW productivity, FW:DW ratios, ash percent, and effective (Y(II)) and optimal (F_m/F_v) quantum yields between nutrient treatments, sediment treatments and harvests (all fixed factors). Winter and summer data were analyzed separately. Tests to assess the assumptions of normality, homoscedasticity, and linearity, appropriate for the models were completed and all assumptions were considered met. Winter and summer data for N removal rate and FAAs in the tissue from the final harvest were analyzed using factorial ANOVAs to test for differences between nutrient treatments, and sediment treatments (all fixed factors), again winter and summer data was analyzed separately. Regression analyses were used to assess the relationships between DW productivity and N removal rate, as well as between productivity and total N removed for each N treatment in both the summer and winter experiments. All analyses were completed in R open-source software (R 4.0.3 GUI 1.73 Catalina build (7892), using r studio (build 443).

2.3 Results

2.3.1 Species identification

Genetic analyses identified 13 of the 23 samples collected as *Gracilaria transtasmanica* (Appendix 2.3). All samples putatively identified as *G. transtasmanica* based on morphological characteristics (Appendix 2.1 & 2.4) described in previous work (Huanel *et al.*, 2020) were confirmed as *G. transtasmanica*. Notably, morphological characteristics of some of the samples confirmed as *G. transtasmanica* differed from those previously recorded

(Appendix 2.1). For example, the thalli of several samples collected during the present study were up to 40 cm in length as opposed to a maximum of 20 cm previously described (Preuss *et al.*, 2020). Samples collected from Little Waihi Estuary and the lower reaches of Maketu Estuary were a mix of *G. transtasmanica* and *G. chilensis*. All samples collected in the upper reaches of the Maketu Estuary beside the Kaituna River outlet (Kaituna Cut) were *G. transtasmanica*.

2.3.2 Growth & productivity

Biomass in all treatments remained healthy and grew throughout the experiments. However, there was a small decrease in productivity in all replicates in the final days of the winter experiment, and some treatments, particularly the low N and low sediment treatments, experienced bleaching in the summer experiment. Low levels of fouling from filamentous species of *Ulva* were present in at least half of all in all replicates by the end of both winter and summer experiments. This fouling largely consisted of fine filaments limited to the lower part of the thalli and was notably greater in the summer experiment than in the winter experiment. Biomass from the summer experiment was somewhat lighter in colour at the beginning of the experiment than the biomass at the beginning of the winter experiment, however the visual differences in colour between the low and high N treatments by the end of the summer experiment was considerable, and all replicates had markedly lighter colour compared to winter samples.

In general, biomass productivity was higher in summer (2.2 g AFDW m^{-2} day^{-1} ± 0.3 S.E. across all treatments) compared to winter (1.1 g AFDW m^{-2} day^{-1} ± 0.1 S.E. across all treatments, Figure 2.3). This difference became more notable with each harvest, as the low

N/low Sediment treatments became more bleached (Figure 2.3). In the summer experiment, biomass productivity varied significantly between sediment treatments and N treatments, however, this variation between N treatments was not consistent among harvests, as evidenced by a significant N treatment x harvest interaction effect (Table 2.1). Biomass productivity in the summer experiment was markedly different between the high and low N treatments (Figure 2.3), ranging from 0.4 g AFDW m^{-2} day^{-1} ± 0.4 S.E. in the low N, no sediment treatment at the final harvest to 4.3 g AFDW m^{-2} day^{-1} ± 0.1 S.E. in the high N, high sediment treatment at the final harvest. Biomass productivity was consistently higher in the high sediment treatments (3 g AFDW m^{-2} day^{-1} ± 0.3 S.E. across all N treatments) compared to the background sediment treatments (2.0 g AFDW m^{-2} day^{-1} ± 0.3 S.E. across all N treatments) which was also higher than the no sediment treatments (1.8 g AFDW m^{-2} day^{-1} ± 0.3 S.E. across all N treatments), and this trend was consistent across all harvests in the summer experiment and became more notable in the second and third harvests.

In contrast to the summer experiment, biomass productivity varied significantly between sediment treatments and harvests in the winter experiment, but not between N treatments (Table 2.1). The biomass productivity in the winter experiment ranged from 0.6 g AFDW m^{-2} day^{-1} ± 0.1 S.E. in the low N, background sediment treatment at the final harvest to 1.8 g AFDW m^{-2} day^{-1} ± 0.1 S.E. in the high N, no sediment treatment at the first harvest treatment. Across all N treatments and harvests, biomass productivity was highest in the no sediment treatments (1.3 g AFDW m^{-2} day^{-1} ± 0.1 S.E.) and was 25% greater than the background sediment loading rate (0.95 g AFDW m^{-2} day^{-1} ± 0.1 S.E.) yet was just 10% greater than the highest sediment loading rate (1.2 g AFDW m^{-2} day^{-1} ± 0.1 S.E.). Across all nutrient and sediment treatments, biomass productivity was 14% lower at harvest two (1.2 g AFDW m^{-2} day^{-1} ± 0.1 S.E.) than harvest one (1.4 g AFDW m^{-2} day^{-1} ± 0.1 S.E.), and 31% lower in harvest three (0.8 g AFDW

m^{-2} day^{-1} ± 0.1 S.E.) compared to harvest two. This meant that across all treatments, biomass productivity was 41% lower in harvest three compared to harvest one. Across all harvests and sediment treatments there was little difference in productivity between the high and low N treatments (1.13 g AFDW m^{-2} day^{-1} ± 0.1 S.E. and 1.12 g AFDW m^{-2} day^{-1} ± 0.1 S.E. respectively).

Table 2.1: Results from analyses of variance (ANOVAs) testing the effects of harvest (Ha), nitrogen (N) and sediment (Se) on AFDW productivity of *Gracilaria transtasmanica* in the winter and summer experiments. Degrees of freedom (df), F values and P values are presented with significant P values in bold.

Season	Effect	df	F	P
Summer	N	1	85.820	**<0.001**
	Se	2	25.491	**<0.001**
	Ha	2	0.292	0.748
	N | Se	2	0.325	0.724
	N | Ha	2	11.793	**<0.001**
	Se | Ha	4	1.112	0.358
	N | Se | Ha	4	0.383	0.820
	Res	72		
Winter	N	1	0.053	0.819
	Se	2	11.231	**<0.001**
	Ha	2	39.587	**<0.001**
	N | Se	2	1.514	0.227
	N | Ha	2	1.930	0.153
	Se | Ha	4	1.796	0.139
	N | Se | Ha	4	2.230	0.074
	Res	72		

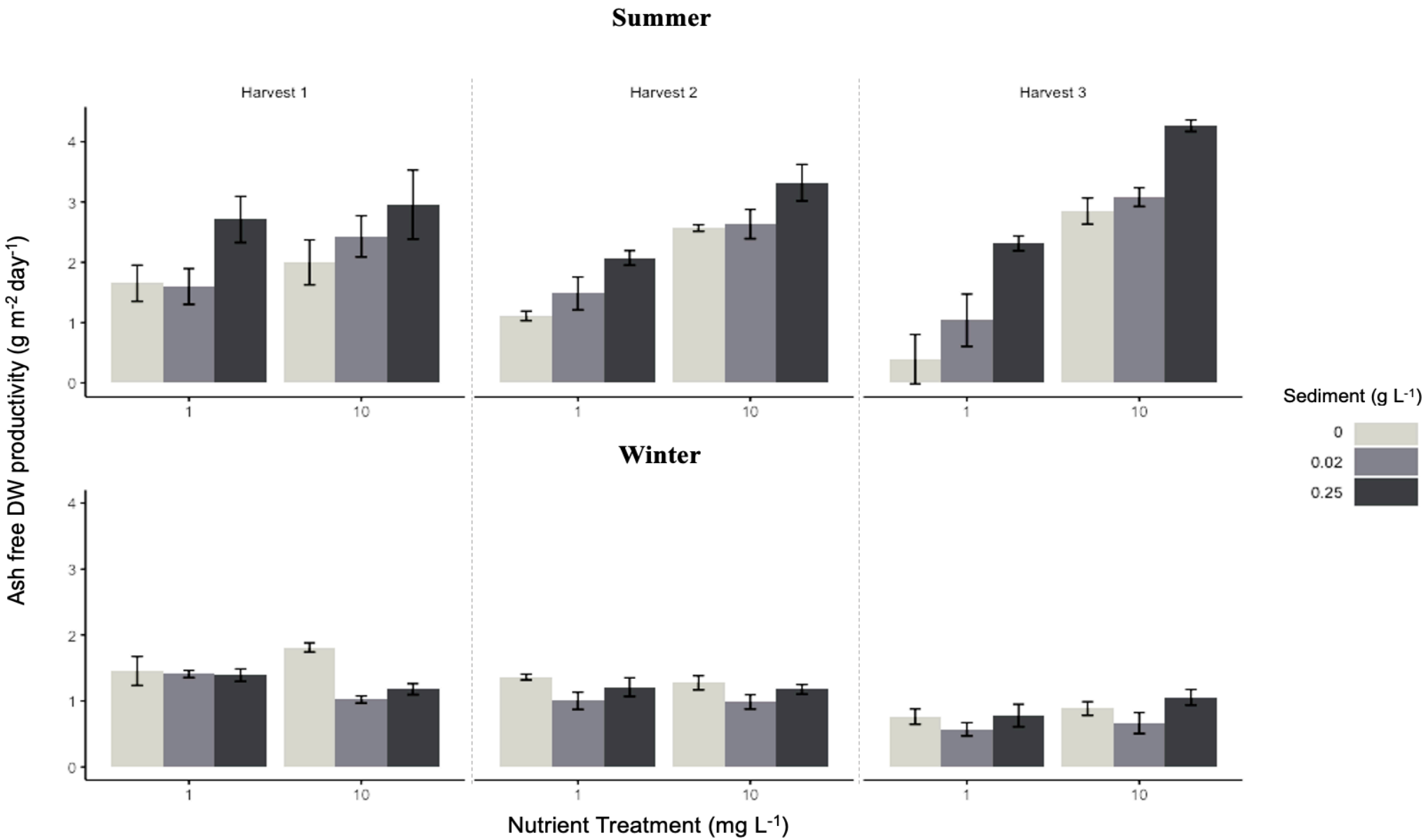

Figure 2.2: Mean (±SE) ash-free dry weight (AFDW) productivity (g m^{-2} day^{-1}) of *Gracilaria transtasmanica* biomass grown under two nutrient treatments (1 and 10 mg N L^{-1}) and three sediment treatments (0, 0.02, and 0.25 g L^{-1}) over three consecutive harvests in the summer (top) and winter (bottom) experiments.

2.3.3 Photosynthetic performance

In the summer experiment, effective quantum yield (Y(II)) was significantly affected by N treatment (Table 2.2, Figure 2.4), Across all harvests and sediment treatments, effective quantum yield was almost 30% higher in the high N treatment (0.3 ± 0.0 S.E.) compared to the low N treatment (0.2 ± 0.0 S.E.). Harvest also had a significant effect on effective quantum yield, which was 30% higher in the first harvest compared to the following two harvests (Figure 2.4). Similarly, optimal quantum yield was also 30% higher in the high N treatments in the summer experiment across all harvests and sediment treatments (0.3 ± 0.0 S.E.) compared to the low N treatments (0.2 ± 0.0 S.E., Figure 2.4). However, these differences were inconsistent across sediment treatments, as evidenced by a significant sediment x N treatment interaction effect (Table 2.2).

Both effective and optimal quantum yields were considerably higher (60% and 50% respectively) in the winter experiment compared to the summer experiment across all treatments and harvests. In the winter experiment, effective quantum yield was significantly different between harvests but not between nutrient or sediment treatments (Table 2.2, Figure 2.4). Across all nutrient and sediment treatments, effective quantum yield was lower in the final harvest (0.4 ± 0.1 S.E.) than the first and second harvests (both = 0.5 ± 0.0 S.E.). Optimal quantum yield was significantly affected by N treatment as well as harvest (Table 2.2, Figure 2.4). Across all treatments, optimal quantum yield decreased as the experiment progressed and was 20% lower on each of the three consecutive harvests. Across all sediment treatments and harvests, optimal quantum yield was also almost 20% higher in the low N treatments (0.42 ± 0.0 S.E.) compared to the high N treatments (0.35 ± 0.0 S.E.) at the final harvest.

Table 2.2: Results from analyses of variance (ANOVAs) testing the effects of harvest (Ha), nitrogen (N) and sediment (Se) on biomass effective quantum yield (Y (II)), and optimal quantum yield (F_m/F_v) of *Gracilaria transtasmanica* in the winter and summer experiments. Degrees of freedom (df), F values and P values are presented with significant P values in bold.

Season	Variable	Effect	df	F	P
Summer	Y (II)	N	1	75.555	**<0.001**
		Se	2	1.047	0.356
		Ha	2	3.947	**0.024**
		N | Se	2	1.316	0.275
		N | Ha	2	0.733	0.484
		Se | Ha	4	0.826	0.513
		N | Se | Ha	4	0.549	0.700
		Res	72		
	F_v/F_m	N	1	50.071	**<0.001**
		Se	2	1.740	0.183
		Ha	2	1.057	0.353
		N | Se	2	3.959	**0.023**
		N | Ha	2	1.112	0.335
		Se | Ha	4	1.572	0.191
		N | Se | Ha	4	1.991	0.105
		Res	72		
Winter	Y (II)	N	1	0.014	0.908
		Se	2	2.705	0.074
		Ha	2	12.772	**<0.001**
		N | Se	2	0.237	0.789
		N | Ha	2	0.821	0.444
		Se | Ha	4	1.853	0.128
		N | Se | Ha	4	0.668	0.616
		Res	72		
	F_v/F_m	N	1	7.455	**0.008**
		Se	2	0.846	0.434
		Ha	2	17.322	**<0.001**
		N | See	2	1.329	0.271
		N | Ha	2	0.621	0.541
		Se | Ha	4	1.179	0.327
		N | Se | Ha	4	1.379	0.250
		Res	72		

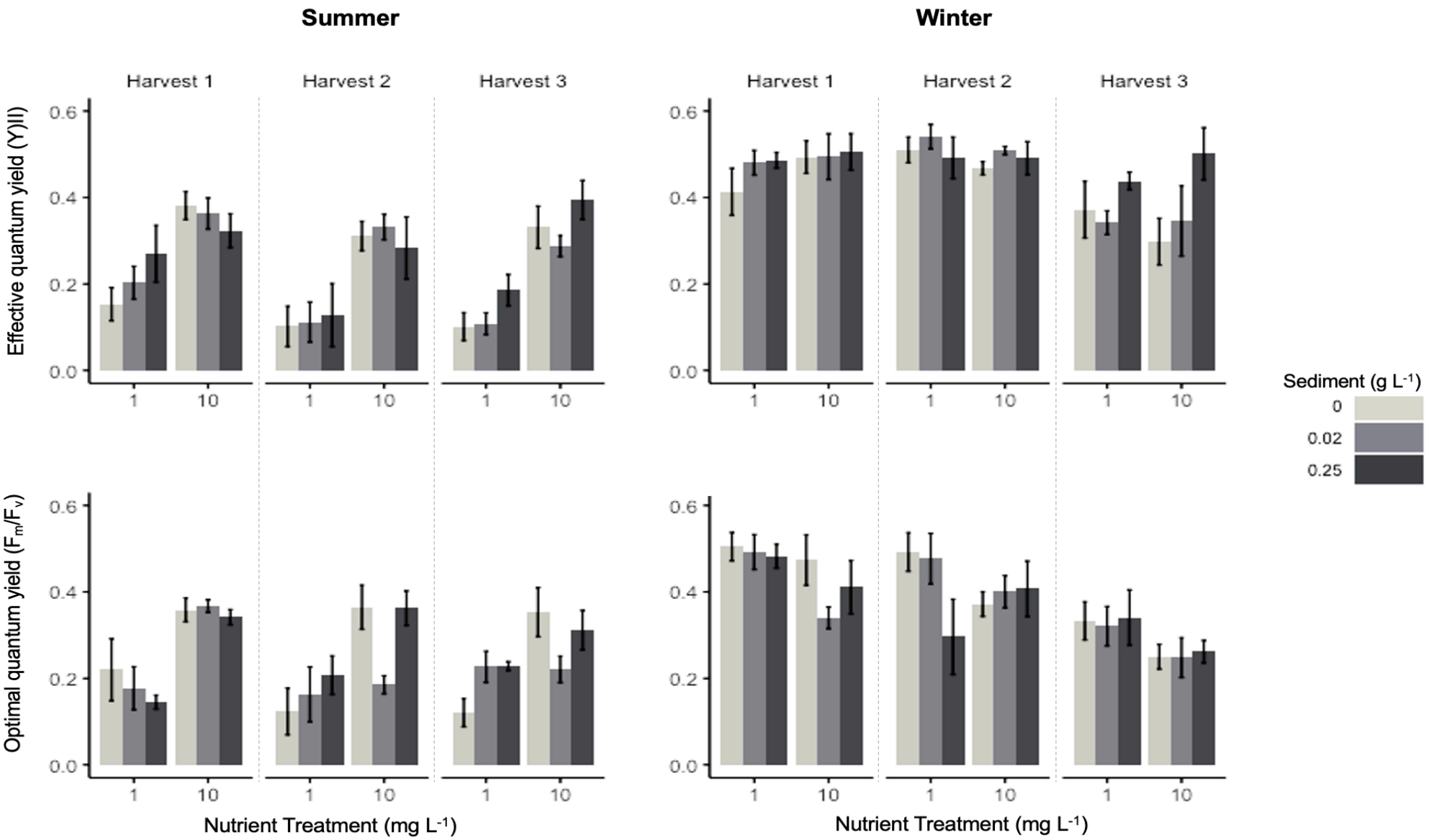

Figure 2.3: Mean (±S.E.) Effective quantum yield (Y(II), top panel); and Optimal quantum yields (F_m/F_v, bottom panel) of *Gracilaria transtasmanica* biomass grown under two nutrient treatments of 1 and 10 mg N L^{-1} and three sediment treatments of 0, 0.02, and 0.25 g L^{-1}, from three harvests, in the summer (left), and winter (right) experiments.

2.3.4 Biomass composition

Biomass FW:DW ratios were lower in the summer experiment than the winter experiment, ranging from 4.1 % ± 0.1 S.E. to 4.3 % ± 0.1 S.E. in summer, and from 4.8 % ± 0.1 S.E. to 5.0 % ± 0.1 S.E. in winter (Table 2.3). Ash content was comparable between seasons but increased with sediment load, and across all N treatments and seasons ranged from 22.6 % ± 0.3 S.E. to 27.05 % ± 0. 4 S.E. (Table 2.3). Carbon content was also comparable between seasons and did not appear to differ with treatment, ranging from 29.9 % ± 0. 4 S.E. to 33.7 % ± 0.1 S.E. across all treatments and seasons (Table 2.3). However, the high sediment treatments had lower carbon contents (31.4 % ± 0.2 S.E.) compared to the low or no sediment treatments (33.4 % ± 0.0 S.E.) across all N treatments. Similarly, hydrogen content was also lower in the high sediment treatments (5.2 % ± 0.2 S.E. across all N treatments), compared to the low or no sediment treatments (both = 5.5 % ± 0.1 S.E. across all N treatments). Hydrogen was lower in the summer experiment (4.7 % ± 0.1 S.E. to 5.3 % ± 0.0 S.E.) compared to the winter experiment (5.4 % ± 0.1 S.E. to 5.9 % ± 0.2 S.E. Table 2.3). The nitrogen content ranged from 3.4 % ± 0.1 S.E. to 4.1 % ± 0.1 S.E. in winter and from 1.6 % ± 0.5 S.E. to 3 % ± 0.1 S.E. in summer (Table 2.3) and was lower in the starting biomass in the summer experiment (1.8 %) compared to the winter experiment (4.5 %). At the end of the 12-day experiment, N content was higher in the high N treatments (4.1 % ± 0.0 S.E.) compared to the low N treatments (3.5 % ± 0.1 S.E.) across all sediment treatments in winter. This trend was also present in the summer experiment with N content ranging from 1.6 % ± 0.1 S.E. in the low N treatment, to 2.9 % ± 0.1 S.E. in the high N treatment. Sulfur content did not appear to be affected by either N or sediment treatment but was higher in the winter experiment compared to the summer experiment, ranging from 2.4 % ± 0.04 S.E. to 2.6 % ± 0.0 S.E. in the winter experiment, and from 2.1 % ± 0.1 S.E. to 2.3 % ± 0.2 S.E. in the summer experiment (Table 2.3).

Table 2.3: FW:DW ratio, ash (%) and ultimate analysis (carbon (C), hydrogen (H), nitrogen (N), and sulphur (S)) (% of DW) of *Gracilaria transtasmanica* biomass from the final harvest of the Winter and Summer experiments. Values are means ± standard error. N = 5 except for initial biomass samples where N = 1. Data reported "as received".

Season	Nutrients	Sediment (g L^{-1})	FW:DW	Ash (%)	C (%)	H (%)	N (%)	S (%)
Summer								
	Initial biomass			30.0	30.1	4.7	1.8	3.5
	1mg L^{-1}	0	4.3 (± 0.1)	23.3 (± 0.3)	33.3 (± 0.1)	5.2 (± 0.1)	1.7 (± 0.2)	2.1 (± 0.1)
		0.02	4.2 (± 0.1)	23.4 (± 0.3)	33.0 (± 0.2)	5.2 (± 0.1)	1.4 (± 0.1)	2.3 (± 0.1)
		0.25	4.1 (± 0.1)	26.3 (± 0.5)	31.0 (± 0.5)	4.9 (± 0.2)	1.6 (± 0.5)	2.3 (± 0.2)
	10mg L^{-1}	0	4.1 (± 0.1)	23.1 (± 0.2)	33.7 (± 0.1)	5.3 (± 0.0)	3.0 (± 0.1)	2.2 (± 0.0)
		0.02	4.1 (± 0.1)	22.9 (± 0.1)	33.5 (± 0.1)	5.2 (± 0.0)	2.9 (± 0.1)	2.3 (± 0.0)
		0.25	4.1 (± 0.1)	27.8 (± 0.5)	29.9 (± 0.4)	4.7 (± 0.1)	2.8 (± 0.1)	2.1 (± 0.1)
Winter								
	Initial biomass			28.4	30.9	5.3	4.5	2.9
	1mg L^{-1}	0	4.8 (± 0.1)	22.3 (± 0.5)	33.4 (± 0.1)	5.7 (± 0.1)	3.4 (± 0.1)	2.5 (± 0.1)
		0.02	4.7 (± 0.1)	22.5 (± 0.2)	33.4 (± 0.1)	5.9 (± 0.2)	3.5 (± 0.1)	2.5 (± 0.1)
		0.25	5.0 (± 0.1)	26.2 (± 0.2)	32.2 (± 0.1)	5.6 (± 0.1)	3.6 (± 0.1)	2.5 (± 0.1)
	10mg L^{-1}	0	4.9 (± 0.0)	24.4 (± 0.3)	33.0 (± 0.1)	5.6 (± 0.0)	4.1 (± 0.1)	2.5 (± 0.0)
		0.02	4.7 (± 0.0)	24.4 (± 0.1)	32.8 (± 0.1)	5.6 (± 0.1)	4.1 (± 0.1)	2.4 (± 0.0)
		0.25	5.0 (± 0.1)	26.3 (± 0.2)	31.8 (±0.2)	5.4 (± 0.1)	4.0 (± 0.0)	2.6 (± 0.0)

2.3.4.1 Nitrogen removal & assimilation

Analysis of water samples from the final harvest of the winter experiment showed a reduction of 0.2 mg N L^{-1} day^{-1} ± 0.0 S.E. and 0.02 mg P L^{-1} day^{-1} ± 0.0 S.E. in the low N treatments, and 0.3 mg N L^{-1} day^{-1} ± 0.1 S.E. and 0.04 mg P L^{-1} day^{-1} ± 0.0 S.E. in the high N treatments across all sediment treatments. Nutrient treatment had a significant effect on P reduction in the culture water ($F_{1,24,} = 12.759$, $P = 0.002$), but not on N reduction ($F_{1,24,} = 1.549$, $P = 0.225$).

Standardised N removal rate (N removed per g DW of growth-1) was 40% lower in summer than winter (Figure 2.4), ranging from 14.4 – 29.8 mg N per g DW of $growth^{-1}$ in summer and 33.8 – 41.4 mg N per g DW of $growth^{-1}$ in winter. In the summer experiment there was a significant difference in standardised N removal rate between the high and low N treatments (ANOVA, $F_{1, 24,} = 107.947$, $P < 0.001$, Figure 2.4), but not between sediment treatments (ANOVA, $F_{1, 24,} = 1.022$, $P = 0.4$). Similarly, there was a difference in standardised N removal rate between N treatments in the winter experiment however this difference was not consistent between sediment treatments, as indicated by a significant N treatment x sediment treatment interaction effect (ANOVA, $F_{1, 24,} = 3.652$, $P = 0.04$). Across all sediment treatments, standardised N removal rate was higher in the high N treatment in both summer (28.7 mg g DW^{-1} ± 0.8 S.E.) and winter (40.7 mg g DW^{-1} ± 0.6 S.E.) compared to the low N treatments (summer: 15.9 mg gDW^{-1} ± 1.7 S.E.; winter: 34.9 mg g DW^{-1} ± 0.7 S.E.). However, in the summer experiment, standardised N removal rate in the low N treatment was almost half that of the high N treatment, whereas in the winter experiment the standardised N removal rate was only slightly lower (15%) in the low N treatment compared to the high N treatment.

Across all treatments, total N removed in the summer experiment was almost 45% higher than the total N removed in the winter experiment (Figure 2.4), ranging from 6 – 30.7 mg N in summer and 6.1 – 9.1 mg N in winter. Total N removed (mg N) in the summer experiment was significantly different between N treatments (ANOVA, $F_{1, 24,} = 144.449$, $P < 0.001$) and sediment treatments (ANOVA, $F_{2, 24,} = 5.517$, $P < 0.01$). However, in the winter experiment there was no difference in total N removed between either N treatments (ANOVA, $F_{1, 24,} = 1.605$, $P < 0.2$) or sediment treatments (ANOVA, $F_{2, 24,} = 0.805$, $P < 0.5$). Across all sediment treatments, total N removed in the summer experiment was considerably higher in the high N treatment (26.5 mg N ± 2.1 S.E.) than in the low N treatment (8.1 mg N ± 2.1 S.E.). In contrast, total N removed in the winter experiment was similar between the high N treatment (8.3 mg N ± 1.0 S.E.) and the low N treatment (7.1 mg N ± 1.1 S.E.) and was comparable to the low N treatment in the summer experiment. Across all N treatments in the summer experiment, total N removed in the high sediment treatment (20.9 mg N), was 25% higher than in both the ambient (15.8 mg N) and the no (15.2 mg N) sediment treatments.

All treatments in the winter experiment had high rates of N removal but low productivity (Figure 2.5a), and regression analysis showed there was no significant relationship between productivity and standardised N removal rate for both the high N treatment ($R= -0.07$, $F_{1,13,} = 0.1233$, $P = 0.7$) and the low N treatment ($R= 0.01$, $F 1_{,13,} = 1.176$, $P = 0.3$). In contrast, in the summer experiment both standardized N removal rate and productivity were high in the high N treatment but were low in the low N treatment (Figure 2.5a). But regression analysis showed that there was no significant relationship between these variables in the high N treatment ($R= -0.4$, $F_{1,13,} = 0.4459$, $P = 0.5$), or the low N treatment ($R= -0.08$, $F_{1,13,} = 0.009034$, $P = 0.9$).

Regression analysis showed that DW productivity was not a good predictor of total N removed for both the high (R= 0.08, $F_{1,13,} = 2.193$, P = 0.16) and low (R= 0.002, $F_{1,13,} = 1.025$, P = 0.3) N treatments in the winter experiment (Figure 2.5b). However, in the summer experiment, there was a strong positive relationship between productivity and total N removed for both the high N treatment (R= 0.4, $F_{1,13,} = 41.17$, P < 0.001), and in the low N treatment (R= 0.4, $F 1_{,13,} = 11.78$, P = 0.005, Figure 2.5b).

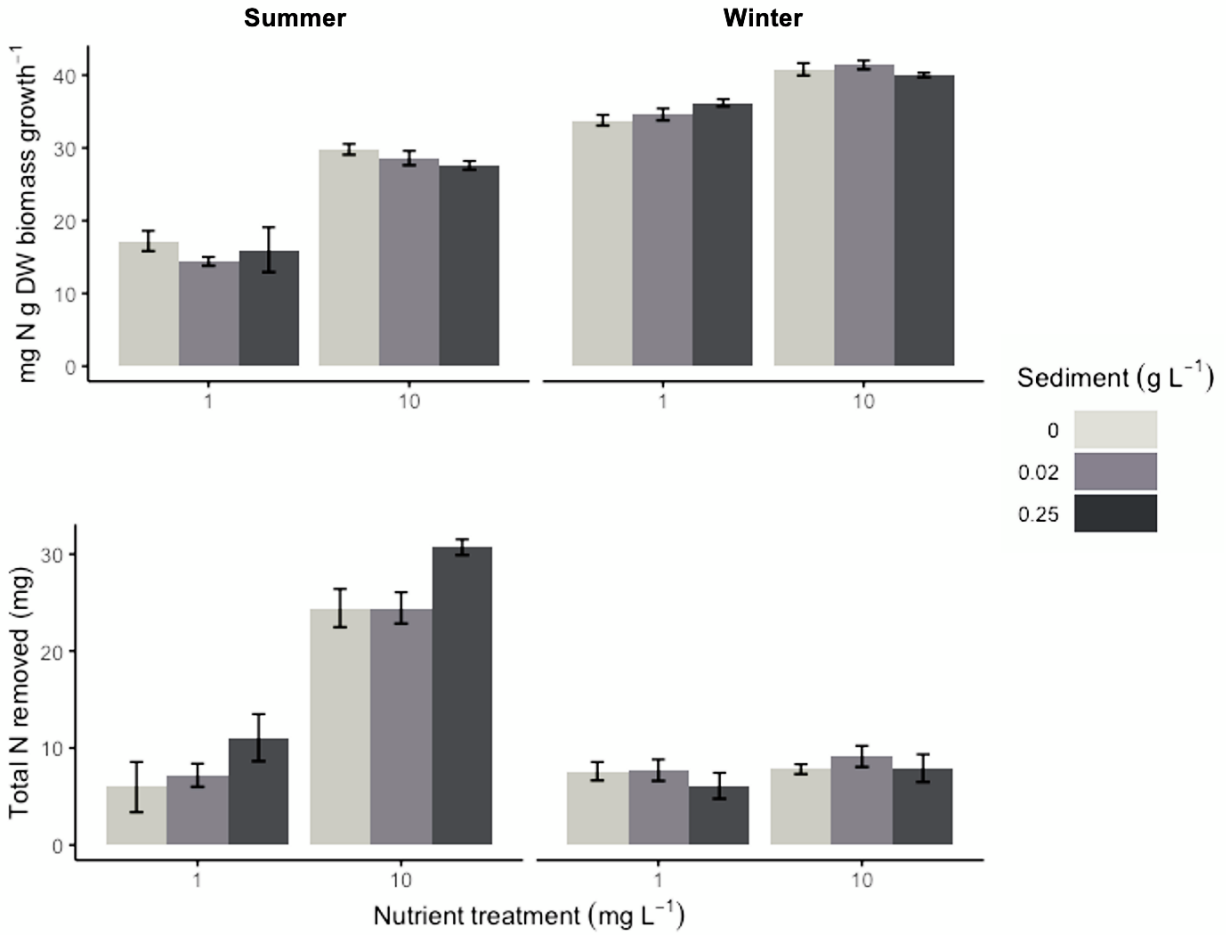

Figure 2.4: Mean (±SE) standardised N removal rates (mg N g DW biomass growth^{-1}) and mean (±SE) total N removed (mg N) by *Gracilaria transtasmanica*, grown under two nutrient treatments (1 and 10 mg N L^{-1}) and three sediment treatments (0, 0.02, and 0.25 g L^{-1}) in the final harvest in both summer (left), and winter (right) experiments.

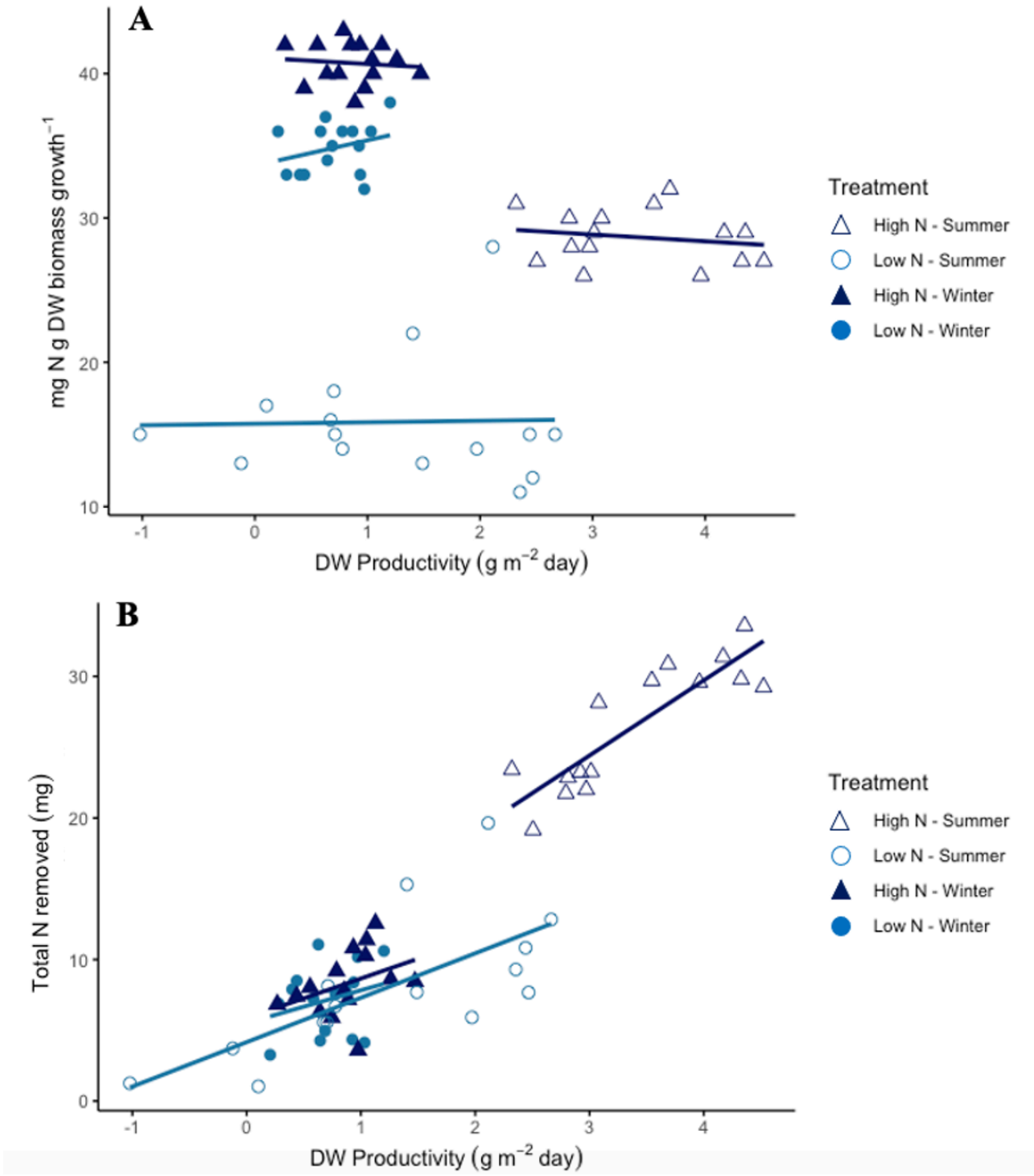

Figure 2.5: Relationships between standardised N removal rates (mg N removed g DW biomass growth^{-1}) and DW productivity (g m-2 day^{-1}) (A), and total N removed (mg N) and DW productivity (g m-2 day^{-1}) (B) by *Gracilaria transtasmanica,* grown under two nutrient treatments (1 and 10 mg N L^{-1}) and three sediment treatments (0, 0.02, and 0.25 g L^{-1}) from the final harvest in both summer and winter experiments. Note that sediment treatments are not separated out in this analysis.

2.3.4.2 FAAs: Gigartinine

Analyses of the calculated FAA content from the biomass samples showed similar trends to N removal rates (Figures 2.5 & 2.6). Free Amino Acids (FAAs) mg per g DW of growth in the summer experiment varied significantly between nutrient treatments (ANOVA, $F_{1, 24}$, = 92.494, $P < 0.001$) but not sediment treatments (ANOVA, $F_{2, 24}$, = 0.552, P = 0.6). Similarly, in the winter experiment FAAs mg per DW g of growth in the winter experiment varied significantly between nutrient treatments (ANOVA, $F_{1, 24}$, = 46.006, $P < 0.001$) but not sediment treatments (ANOVA, $F_{2, 24}$, = 1.112, P = 0.4). Across all sediment treatments, FAAs mg per g DW of growth were 35% lower in the low N treatment in summer (16.7 mg g DW^{-1} ± 1.8 S.E.) compared to the low N treatment in winter (26 mg g DW^{-1} ± 2.6 S.E.). However, FAAs were 23% higher in the high N treatment in summer (55.3 mg g DW^{-1} ± 6.3 S.E.) than the high N treatment in winter (42.8 mg g DW^{-1} ± 2.8 S.E., Figure 2.6). In the summer experiment, mg FAAs per g DW growth were 70% higher in in the high N treatments compared to the low N treatments, whilst in the winter experiment the FAAs mg per g DW growth was 40% higher in the high N treatments than the low N treatments.

Total FAAs (not standardized by growth) also varied significantly with N treatment in both the summer experiment (ANOVA, $F_{1, 24}$, = 108.006, $P < 0.001$) and in the winter experiment (ANOVA, $F_{2, 24}$, = 11.668, P = 0.002, Figure 2.6). Moreover, sediment also had no effect on FAAs in the summer experiment (ANOVA, $F_{2, 24}$, = 2.245, P = 0.1) or in the winter experiment (ANOVA, $F_{2, 24}$, = 0.74, P = 0.8). Across all sediment treatments, total FAAs were 30% higher in the low N treatment in summer (7.8 mg ± 2.0 S.E.) than the low N treatment in winter (5.4 mg ± 1.1 S.E.) and were 83% higher in the high N treatment in summer (51.6 mg ± 1.1 S.E.) than the high N treatment in winter (8.6 mg ± 1.1 S.E. Figure 2.6). Total FAAs were 85% higher in the high N treatments

compared to the low N treatments in the summer experiment but were only 37% higher in the high N treatments compared to the low N treatments in the winter experiment.

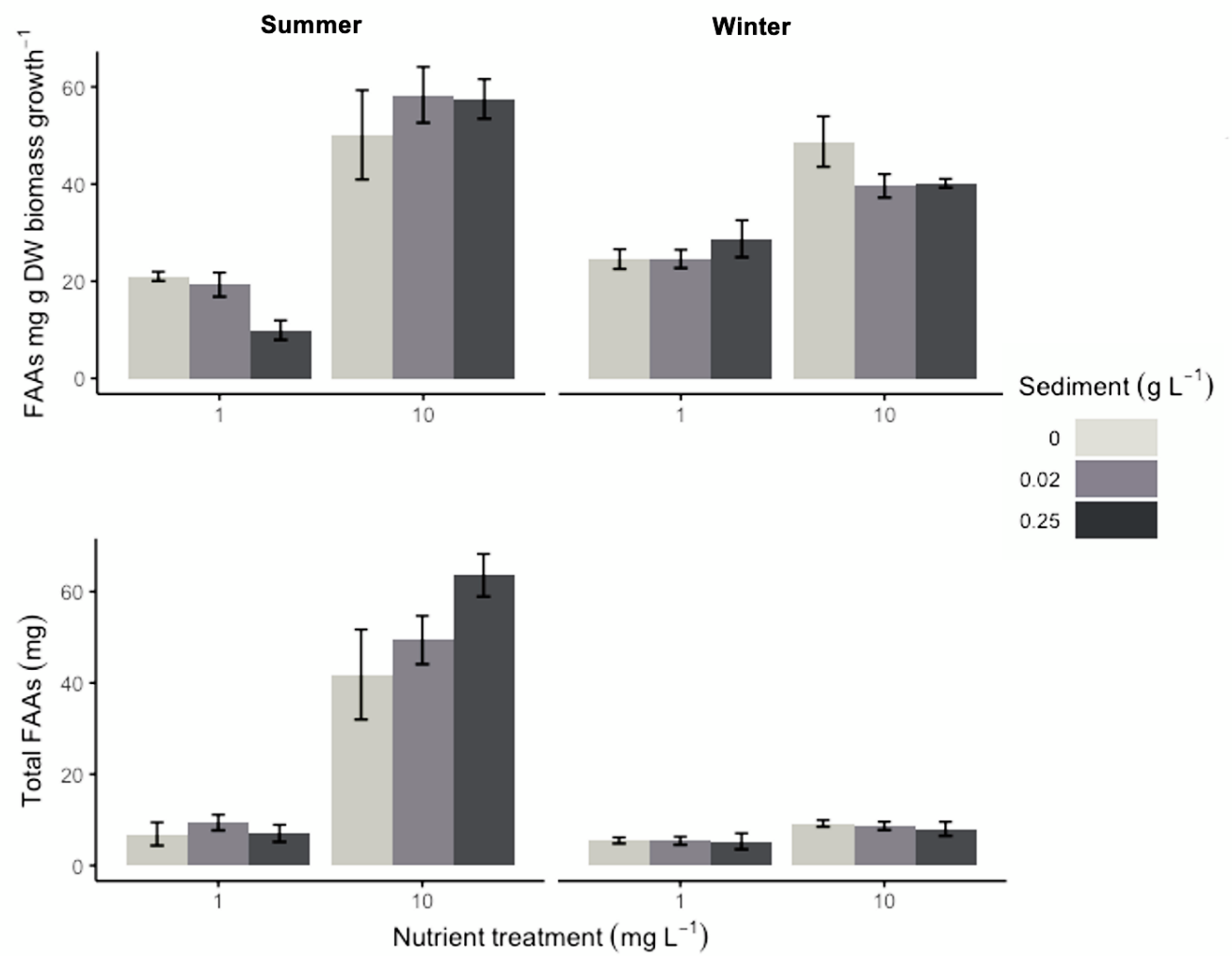

Figure 2.6: Mean (±SE) FAAs (mg g DW biomass growth^{-1}) and mean (±SE) total FAAs (mg FAAs) in the tissue of *Gracilaria transtasmanica* grown under two nutrient treatments (1 and 10 mg N L^{-1}) and three sediment treatments (0, 0.02, and 0.25 g L^{-1}) in the final harvest in both summer (left), and winter (right) experiments.

2.4 Discussion

In situ macroalgal bioremediation is a promising solution to nutrient loading in enriched estuaries (Fei, 2004; Kim *et al.*, 2014; Kim *et al.*, 2015; Rose *et al.*, 2015; Seghetta *et al.*, 2016). A critical first stage in developing macroalgal bioremediation for estuarine restoration is ensuring target species for cultivation can assimilate nutrients under

relevant local conditions (Lawton. *et al.*, 2013; Rose *et al.*, 2015). Novel macroalgal species for aquaculture are typically selected based on biomass productivity and composition (Lawton. *et al.*, 2013; Roleda & Hurd, 2019; Lawton *et al.*, 2021b). As an alternative approach, we used bioremediation potential as the primary criterion to determine the suitability of *Gracilaria transtasmanica* as a novel target species (de Oliveira *et al.*, 2016; Gentry *et al.*, 2020). Our experiments demonstrated that *G. transtasmanica* can effectively assimilate nitrogen under ambient summer and winter conditions, even when biomass productivity is low. Furthermore, *G. transtasmanica* can assimilate nutrients under a range of environmental conditions that are representative of both local background and high levels of suspended sediments and nutrient concentrations.

Biomass productivity was considerably higher in summer than winter; with as much as a 4-fold increase in in some treatments. This result matches findings from seasonal studies with other species of *Gracilariales*, which also show higher growth in summer relative to winter conditions (Abreu *et al.*, 2011). Higher productivity across most treatments in summer is likely reflective of the at least 4-fold increase in PAR received by cultures in summer compared to winter. However, optimal and effective quantum yields showed that the biomass was less stressed and generally in better health in the winter experiment, with limited damage to photosystem II in all treatments. Differential biomass productivities in the summer and winter experiments may also have been influenced by a varying effect of sediment treatments. High sediment treatments, causing elevated turbidity, had a negative impact on productivities in winter, but a positive impact in summer. This effect is likely a result of sediment creating a shading effect which reduced light reaching the algae in winter, thereby reducing productivity, but protected it from the extreme light levels in summer, thus reducing stress and increasing productivity (Häder & Figueroa, 1997;

Häder *et al.*, 1998; Mata *et al.*, 2006). Supporting this hypothesis, high sediment treatments in the summer experiment had higher effective and optimal quantum yields, indicative of limited cell damage, compared to the low sediment treatments. Moreover, the bleaching observed in some replicates in the low N/low sediment treatments in summer coincided with low productivity rates, and lower effective quantum yield and optimal quantum yields in these treatments suggests the functioning of photosystem II was negatively impacted. These results suggest that a higher stocking density may be required to maintain healthy biomass condition under summer conditions as a higher stocking density typically causes self-shading of the biomass and therefore reduces the amount of light the algae biomass receives (Carpenter, 1990; Mata *et al.*, 2006; Higgins *et al.*, 2008). Alternatively, optimal in-situ growth of *G. transtasmanica* may be sustained in summer months by shifting cultivation lines to deeper water as this is likely to reduce the intensity of irradiance (Kim *et al.*, 2014). Further studies exploring the relationships between culture density, productivity, and cell health under high light conditions will provide clarity for these results (Mata *et al.*, 2006; Abreu *et al.*, 2011).

Biomass productivity is generally used as a proxy for bioremediation ability, with a positive correlation assumed between these variables (Neori *et al.*, 2004; Barrington *et al.*, 2009; Abreu *et al.*, 2011). However, *G. transtasmanica* showed the highest rates of N assimilation per g DW growth when it had the lowest productivities. Moreover, despite low productivity in winter compared to summer, FAA content (reflective of gigartinine content) in both low and high N treatments was relatively high, further showing capacity for N uptake independent of growth. This is a desirable trait in a target species for bioremediation as it means that *G. transtasmanica* can maintain N removal in both optimal and sub-optimal conditions (Rose *et al.*, 2015). In contrast to the winter experiment, *G. transtasmanica* had high N removal rates and high productivity in the high

N treatments in the summer experiment. This finding suggests that when conditions are optimal for growth, the majority of N taken up by the biomass will be assimilated directly into biomass growth. However, the high N treatments in summer also showed a considerable increase in FAA content, suggesting that *G. transtasmanica* can actively take up and assimilate luxury N and convert it to storage as gigartinine under high light, high nutrient conditions. This result corroborates previous findings showing that *G. transtasmanica* has increased gigartinine concentrations when cultivated under high nutrient conditions (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Huanel *et al.*, 2020). Overall, these results were comparable to findings reported for other species of *Gracilaria,* where N removal rates were lower during the winter months but elevated in summer as light and temperature increased (Abreu *et al.*, 2011; Kim *et al.*, 2014). These results show that *G. transtasmanica* has high potential for year-round N removal.

To be an effective species for bioremediation, target species must be able to maintain consistent nutrient assimilation under dynamic local conditions (Lawton. *et al.*, 2013; Rose *et al.*, 2015). *Gracilaria transtasmanica* demonstrated this ability in all treatments tested, which represent both background local conditions and extreme turbidity and high nutrient conditions. Nitrogen and phosphorus removal in winter showed the species to be effective in bioremediation during sub-optimal light and temperature conditions. However, in addition to effective N assimilation and sustained growth, an ideal target species for bioremediation should also experience limited fouling and be robust in variable environmental conditions (Lawton. *et al.*, 2013; Lawton *et al.*, 2021b). In the present study *G. transtasmanica* suffered ongoing issues in some treatments with fouling in the summer experiment, in addition to highly varied N assimilation and elevated photoinhibition. Such issues can be common in small scale research trials investigating the suitability of novel species for cultivation (Mata *et al.*, 2006; Roleda & Hurd, 2019;

Toomey *et al.*, 2020). However, both photoinhibition and fouling could potentially be addressed through increased stocking density or altered culture systems (Abreu *et al.*, 2011) and may be less of an issue when biomass is cultivated in-situ rather than under small scale batch cultures as in the current experiment. Nevertheless, the findings outlined here suggest that macroalgal bioremediation with *G. transtasmanica* could provide a novel approach to successfully reducing nutrient enrichment and eutrophication in estuarine habitats.

In addition to being able to assimilate nutrients, target species for bioremediation should also have potential for biomass applications once harvested (Lawton. et al., 2013; Rose et al., 2015; Saldarriaga-Hernandez et al., 2020; Lawton et al., 2021a). The elemental composition of biomass has a considerable influence on its suitability for varying end-product applications (Ebeling & Jenkins, 1985; Lawton. et al., 2017; Lawton et al., 2021b). The relatively high N content in the biomass (up to at least 4%) suggests that G. transtasmanica grown in nutrient enriched water could be a valuable protein-rich food source for aquaculture and agricultural purposes (Aitken & Senn, 1965; Naidoo et al., 2006; Cian et al., 2019; Øverland et al., 2019; Salcedo et al., 2020) or an alternative source of protein for a growing human population (García-Vaquero & Hayes, 2016; Ashokkumar et al., 2022). Using a universal nitrogen-to-protein conversion factor of 5 (Angell et al., 2016), the crude protein content of biomass in the high N treatments in the winter experiment was 20% DW. This value is high compared to reported mean crude protein contents in red algae reported in previous studies (mean of 13.3% DW, Angell et al., 2016), but is likely influenced by a high content of non-essential storage amino acid (e.g., gigartinine) in G. transtasmanica. The presence of gigartinine in G. transtasmanica likely represents a large proportion of the FAA contents reported here (Wilcox et al., 2001; Wilcox et al., 2007) (see Section 2.3.4.2), and showed similar trends to the N contents of

the biomass, with total FAAs being considerably higher in the high N treatments compared to the low N treatments in the summer experiment and also compared to all treatments in the winter experiment. This is also reflective of the known conversion of N to gigartinine in the biomass (Huanel et al., 2020; Preuss et al., 2020). Notably, large differences in the initial (start of experiment) nitrogen content of biomass between seasons (N content was more than 50% lower in summer compared to winter) were most likely due to the fact that stock cultures were maintained under considerably lower (75%) nutrient concentrations prior to the commencement of the summer experiment to reduce fouling. This meant that the initial biomass used to stock the summer experiment had a lower N content than the initial biomass using to stock the winter experiment, and these differences most likely persisted throughout the experiments.

The suitability of macroalgal biomass for potential end-use applications is also impacted by the FW:DW ratio and ash content (Ebeling & Jenkins, 1985; Cole *et al.*, 2015; Lawton. *et al.*, 2017; Lawton *et al.*, 2021a). The FW:DW ratios were not affected by nutrient or sediment treatments in either season, however, they were relatively high compared to the aquaculture prospect *Ulva stenophylloides* (previously *Ulva. Sp. B.,* Lawton *et al.*, 2021b). Whilst maintaining a consistent and predictable FW:DW ratio is a useful characteristic for maintaining reliable biomass production, a low FW:DW ratio is generally preferred as this is indictive of lower water content, which in turn reduces effort required to dry biomass for processing (Ebeling & Jenkins, 1985). Therefore, the lower FW:DW ratio in summer as opposed to winter is a desirable characteristic (Ebeling & Jenkins, 1985). This lower FW:DW ratio in the summer experiment may be linked to higher rates of evaporation in cultures, increasing the salinity and reducing the water content in the cells of the biomass. Therefore, FW:DW ratios may be directly affected by the location where biomass is cultivated as salinity can vary widely within estuarine

habitats (Kamer & Fong, 2000). Ash content was significantly affected by sediment treatment, with the high sediment treatments having up to 4% more ash after 12 days, despite rinsing the biomass prior to harvesting to remove any lightly attached sediment particles. This result suggests some particles of sediment and/or mineral matter introduced by the sediment were taken up by the biomass. High ash content can be favorable in biomass used for feeds as it is indicative of a high content of minerals that are essential for animal nutrition (Cole *et al.*, 2015). In combination, these results suggest biomass of *G. transtasmanica* cultivated in nutrient rich and turbid water will be suitable for a broad range of biomass applications.

2.4.1 Conclusion

Gracilaria transtasmanica is a promising candidate for in-situ algal bioremediation in nutrient rich and turbid New Zealand estuaries. An ability to maintain year-round productivity, robustness in challenging and dynamic conditions, and high N assimilation potential are all necessary characteristics in a target species for bioremediation (Lawton. *et al.*, 2013; Rose *et al.*, 2015). This study showed that *Gracilaria transtasmanica* meets these requirements. The next step to domesticating this species for cultivation will be to identify the environmental conditions that optimise growth (Toomey *et al.*, 2020). The environmental thresholds and optimal range of *G. transtasmanica* for factors such as salinity, turbidity, and exposure to air are currently unknown and will therefore be critical to determine.

Chapter 3

Tolerance of *Gracilaria transtasmanica* to abiotic estuarine conditions

3.1 Introduction

Estuaries are under increasing anthropogenic stress due to nutrient enrichment (Howarth, 2008; Stewart *et al.*, 2018; Malone & Newton, 2020; Plew *et al.*, 2020). This nutrient enrichment is leading to widespread eutrophication in many estuarine ecosystems (Plew *et al.*, 2018b; Malone & Newton, 2020). In-situ macroalgal bioremediation – the cultivation of live macroalgae directly in nutrient-enriched waters for the primary purpose of nutrient assimilation – could help prevent and reduce estuarine eutrophication (Fei, 2004; Kim *et al.*, 2014; Rose *et al.*, 2015; Kim *et al.*, 2015; Seghetta *et al.*, 2016). However, estuaries are challenging and dynamic ecosystems (Green, 2006; Statham, 2012) that are influenced by both terrestrial and oceanic impacts due to their location at the land-sea interface (Kennish, 2002; Statham, 2012). Varying environmental conditions in estuaries can limit the productivity of macroalgal species (Mangan *et al.*, 2020b), and thus may impact bioremediation potential (Barrington *et al.*, 2009; Abreu *et al.*, 2011). Therefore, when selecting novel target species for in-situ bioremediation, assessing the environmental tolerance of the target species to abiotic conditions is crucial to ensure that healthy biomass and bioremediation efficiency can be maintained under relevant local conditions (Abreu *et al.*, 2011; Lawton. *et al.*, 2013; Lawton *et al.*, 2014; Rose *et al.*, 2015; Roleda & Hurd, 2019).

Estuaries are characterized by fluctuations in abiotic conditions such as salinity, exposure to desiccation, and turbidity which causes light limitation (Dawes *et al.*, 1998; Kennish, 2002; Mata *et al.*, 2006; Abreu *et al.*, 2011; Karsten, 2012; Statham, 2012; Holzinger & Karsten, 2013; Scherner *et al.*, 2013; Coppede Cussioli, 2018; Gall *et al.*, 2019; Malone & Newton, 2020; LINZ, 2022). Variation in these factors can have considerable impacts on macroalgal photosynthetic performance, biomass growth, and bioremediation (Dawes *et al.*, 1998; Mata *et al.*, 2006; Abreu *et al.*, 2011; Karsten, 2012; Holzinger & Karsten, 2013; Scherner *et al.*, 2013). Salinity can be highly variable in coastal waters and is affected by freshwater flow, particularly during storm events with high rainfall, and tidal dynamics (Kamer & Fong, 2000; Statham, 2012; Karsten, 2012; Scherner *et al.*, 2013). Salinity affects osmotic balance in macroalgal cells, and extreme levels of high or low salinity can cause stress and consequently impact productivity (Dawes *et al.*, 1998; Karsten, 2012; Holzinger & Karsten, 2013). However, osmotic acclimation is possible with time, and many seaweeds in brackish estuarine waters can utilize ion transport and biosynthesis as mechanisms to cope with salinity flux (Karsten, 2012; Kumar *et al.*, 2014). Tidal cycles cause varied levels of exposure in macroalgae inhabiting intertidal zones, resulting in desiccation stress which can be exacerbated if exposure occurs during periods of high light and heat (Mata *et al.*, 2006; Gray *et al.*, 2007; Holzinger & Karsten, 2013; Zamir *et al.*, 2018). This stress directly inhibits cellular functioning, and therefore productivity, through photoinhibition as a result of extreme irradiance, potentially causing both short- and long-term damage which can be detrimental if on-going (Häder & Figueroa, 1997; Häder *et al.*, 1998; Mata *et al.*, 2006). Sedimentation via freshwater runoff and riverine discharge can increase turbidity, and thereby increase light limitation (Gall *et al.*, 2019; Mangan *et al.*, 2020b; Blain *et al.*, 2021). This light limitation can reduce the rate of photosynthesis and therefore growth (Serôdio *et al.*, 2008; Gall *et al.*, 2019; Mangan *et al.*, 2020b; Blain *et al.*, 2021).

Gracilaria is a genus of *Rhodophyta* with a global distribution across a broad range of habitats (Karsten, 2012; Kim *et al.*, 2014; Rose *et al.*, 2015; Preuss *et al.*, 2020). Currently, two species of *Gracilaria* - *G. chilensis* and *G. tenuistipitatum* - are intensely cultivated internationally and have a combined annual production volume of 3.9 million tonnes (Rose *et al.*, 2015; Preuss *et al.*, 2020). The species *Gracilaria transtasmanica* is indigenous to New Zealand and occurs in estuaries throughout the country (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Huanel *et al.*, 2020; Preuss *et al.*, 2020). Like most of the *Gracilariales, G. transtasmanica* is a versatile species found in a broad range of habitats (Wilcox *et al.*, 2001; Wilcox *et al.*, 2007; Huanel *et al.*, 2020; Preuss *et al.*, 2020; Nelson *et al.*, 2021). *Gracilaria transtasmanica* has recently been identified as a novel target for in-situ estuarine bioremediation due to its broad distribution and apparent tolerance to wide variations in salinity, exposure, desiccation, nutrient supply, and turbidity (Huanel *et al.*, 2020; Preuss *et al.*, 2020; Dudley *et al.*, 2022). Additionally, there is potential to fast-track domestication of this species using cultivation protocols established for *G. chilensis*, *G. tenuistipitatum,* and *G. tikvahiae* (Yarish *et al.*, 2012; Preuss *et al.*, 2020). *Gracilaria transtasmanica* is effective at assimilating N and P under a range of conditions, with nutrient removal rates being highest if healthy biomass is maintained (Chapter Two). However, the optimal conditions for growth and tolerance to varying estuarine environmental conditions have not yet been identified for this species. This is the necessary next step to determine the suitability of this species for in-situ estuarine bioremediation.

The aim of this chapter is to investigate the tolerance of *G. transtasmanica* to abiotic estuarine conditions. The specific aims are to determine the effects of low salinity, desiccation relevant to low-tide exposure, and turbidity and associated light limitation on biomass growth and photosynthetic cell health. Each of these factors will be tested separately in controlled experiments to provide an understanding of the optimal environmental conditions for culturing

G. transtasmanica. This will enable optimal habitat types within tidal and salinity gradients to be determined, and consequently, enable sites within estuaries that maximise productivity and associated bioremediation performance of *G. transtasmanica* to be identified.

3.2 Methods

Gracilaria transtasmanica stock cultures established from wild stocks (described in Chapter Two, Section 2.2.1) were utilised for these experiments. Stock cultures were maintained in 20 L plastic buckets with filtered nutrient-enriched (Varicon Aqua Cell-Hi F2P, 3.075 mg N L^{-1}, and 0.275 mg P L^{-1}) seawater and provided with gentle aeration in an environmentally controlled room (18ºC, 12:12 light:dark cycle, at 200 µmol photons m^{-2} sec^{-1} using cool fluorescent lights). The salinity of the seawater was manually adjusted to 25 ppt by adding filtered dechlorinated freshwater to each bucket. Stock cultures were maintained at this salinity as it is comparable to the salinity of estuarine habitats where *G. transtasmanica* biomass was collected from to establish stock cultures. Additionally, growth appeared more consistent, and fouling was lower at this salinity than when cultures were maintained under 35 ppt. Stock cultures were continuously maintained and used to supply biomass for each experiment.

3.2.1 Experimental design

Three consecutive four-week experiments were conducted to assess the tolerance of *G. transtasmanica* to abiotic conditions that frequently occur in estuaries. Each experiment had four treatments, with five replicates of each treatment. The first experiment investigated salinity and tested treatments of 35, 25, 15, & 5 ppt. These values were selected as *G.*

transtasmanica is an estuarine species targeted for culture in brackish waters. Therefore, these salinity treatments represented the range of salinities found in estuaries, ranging from ocean water (35 ppt, control) to fresh water (0 ppt). The salinity of the seawater was manually adjusted by adding filtered dechlorinated freshwater to filtered seawater to create 12 L^{-1} stock solutions of each desired salinity treatment. The salinity of each stock solutions was confirmed with an ATC Salinity Refractometer prior to use.

The second experiment investigated exposure periods resulting from tidal ranges frequently experienced in New Zealand estuaries (Ellis *et al.*, 2000; LINZ, 2022). Exposure periods of 0 hours (control), 3 hours, 6 hours, and 9 hours were selected. These treatments were based on the range of exposure times that biomass would be subjected to if it was cultivated in low intertidal (3 hours), mid intertidal (6 hours, assumed standard tidal period) and upper intertidal (9 hours, location specific extreme tides) estuarine habitats (LINZ, 2022). The tidal exposure periods were implemented using pre-set Jackson 24hr Mechanical Timers on the pumps of the five replicates of each treatment. These tidal exposure periods were repeated twice in each 24-hour period and the cycle was shifted forward 3 hours every 3 – 4 days to replicate natural tidal cycling.

The third experiment assessed turbidity using shading to create light limitation as a proxy for turbidity. Treatments were selected based on local ambient conditions and possible extreme values. Measurements taken at Little Waihi Estuary, where biomass used to establish stock cultures was originally collected from (Chapter 2, Section 2.2.1), indicated that ambient irradiance loss on existing *G. transtasmanica* beds was 20 – 30 % of surface irradiance. These measurements were taken at the sediment surface on a clear, bright day, with calm conditions at high tide when the shallow mudflats were fully submerged in at least 30cm of water. Local

estuaries can experience extreme weather events and an additional 45% of irradiance can be lost in storm events (Coppede Cussioli, 2018; Gall *et al.*, 2019). Furthermore, in some coastal zones, turbidity pulses can cause extreme and sudden low/no-light conditions that last for a short period of time, known as a pulse effect (Babuder, 2021). Therefore, treatments consisted of shade levels that reduced irradiance by 0% (control), 25% (ambient), 75% (high ambient plus an additional 45% irradiance loss representing a large storm event), and a pulse treatment of full blackout for 2 days, followed by a gradual increase in irradiance: 1 day 50% loss, 1 day 25% loss, and 3 days no loss. This pulse treatment cycle was repeated each week for the full 4-week experiment. The shade treatments were implemented using wax paper, mesh cloth, and blackout covers to create the required loss of irradiance for each treatment. The control treatments received an estimated 250 – 275 μmol photons m^{-2} sec^{-1} at the surface of the water. The light received by the 0%, 25%, and 75% irradiance loss treatments was calculated to be 151.2, 113.4, and 37.7 mol photons m^{-2} $week^{-1}$ respectively. The pulse treatment received 91.8 mol photons m^{-2} $week^{-1}$, equating to a 60% loss of irradiance per week relative to the control treatment. Throughout all three experiments, all factors other than the specific factor being tested were kept constant, e.g., during the salinity experiment all replicates were kept under full submersion and without shade. Additionally, a control treatment was maintained throughout all three experiments with no light limitation, 0 hours exposure, and 25 ppt salinity (assumed optimal conditions).

3.2.2 Biomass Productivity and tolerance

All three experiments were carried out with microFANS (micro–Filamentous Algae Nutrient Scrubbers) systems that were engineered to be run with the varying salinities, irradiance levels, and exposure periods required for these experiments. MicroFANS systems are individual

recirculating systems that can be used to test the performance of macroalgae under continuously flowing water (Hariz *et al.*, 2022). Each microFANS is a stand-alone system consisting of a 2L capacity sump tank filled with nutrient enriched filtered seawater that is continuously recirculated and pumped over the surface of a biomass grow bed using an Aquaforce QD-1900 pump (Figure 3.1). Protocols and methods for each experiment were identical, except for the differing treatments as described above. Nutrients were dosed into each microFANS replicate twice per week at concentrations that equated to local environmental background rates (Chapter Two, Section 2.2.3., Scholes, 2015) of 0.3 mg N L^{-1} day^{-1} (supplied as sodium nitrate $NaNO_{3-}$) and 0.03 mg P L^{-1} day^{-1} (supplied as sodium phosphate $H_2NaPO_4.2H_2O$). Five g fresh weight (FW) biomass was placed on the biomass grow bed of each replicate microFANS system, equating to a stocking density of 2.5 g FW per L of seawater. The 20 replicate systems were arranged in a randomised block design in an environmentally controlled room (18ºC, 12:12 light:dark cycle, at 250 - 275 µmol photons m^{-2} sec^{-1} using cool fluorescent lights), and replicates were rotated twice weekly within each block. Water evaporation was checked regularly, and replicates were sampled randomly throughout the experiments to check salinity levels with an ATC Salinity Refractometer. Sump tanks were topped up with filtered fresh water as required to maintain target salinity treatments.

The biomass in each microFANS was harvested once each week. Biomass was removed from each microFANS and placed into individual mesh bags, spun in a centrifugal spin dryer (Spindel dryer SPL65) for 3 minutes to remove excess water, and then weighed to measure growth. Five g FW of the harvested biomass was then returned to the same replicate microFANS to restock and water in the sump tank of each microFANS was replaced with fresh filtered nutrient-enriched seawater. Excess harvested biomass not restocked back into the microFANS was weighed, placed into pre-weighed falcon tubes and dried on-site in a freeze

dryer (*BUCHI LyovaporTM* L-200 Freeze Dryer), and then reweighed to obtain the dry weight (DW). The fresh weight to dry weight ratio (FW:DW) was then calculated for each individual replicate and used to convert FW growth into DW growth. This process was repeated each week over four consecutive weeks for each of the three experiments. Specific growth rate (SGR, % day^{-1}) was calculated for each replicate microFANS for each harvest for each experiment using the formula, $Ln(Bf/Bi)/T * 100$: where Bf and Bi are the final and initial biomass (g DW) and T is the number of days in culture.

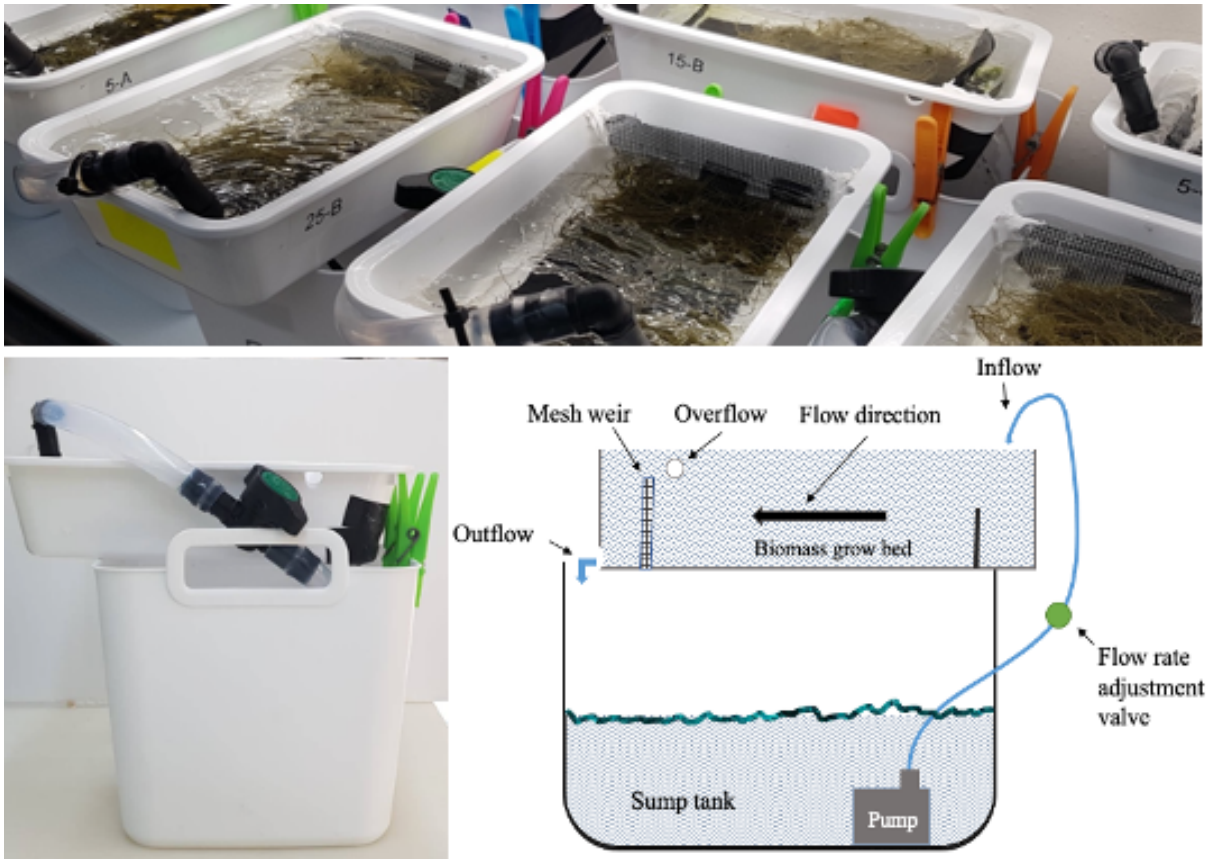

Figure 3.1: Top view of microFANS systems showing water flowing over biomass placed on the biomass grow bed during the salinity experiment (top); profile view of the microFANS system (bottom left); and schematic side view of the microFANS system (bottom right).

3.2.3 Photosynthetic performance

Pulse Amplitude modulation (PAM) measurements were used to assess the impacts of each treatment on the photosynthetic performance of the algae, particularly the health of photosystem II (Chapter Two, Section 2.2.4) in each experiment. Effective quantum yield (Y(II)) and optimal quantum yield (F_v/F_m) were measured each day for first seven days of each experiment to capture fine scale effects during the probable acclimation period, and then weekly on each harvest day for the remaining three weeks of each experiment. The measurements were taken between 8 and 10 am on each occasion, and on harvest days measurements were taken before the harvesting process commenced. Optimal quantum yield (F_v/F_m) measurements were taken following the placement of biomass in complete darkness for a minimum of ten minutes.

3.2.4 Statistical Analysis

Statistical results are presented as the mean ± S.E. Factorial ANOVAs were used to test for differences in SGR and FW:DW ratios in each experiment, with harvest and treatment as fixed factors. Effective (Y(II)) and optimal (F_m/F_v) quantum yields were analyzed similarly, however harvest was replaced with days in culture; treatment levels remained the same. Data from each experiment (low salinity, tidal exposure, light limitation) were analyzed separately. Assumptions of normality, homoscedasticity, and linearity appropriate for all models were tested and considered met. All analyses were completed in R open-source software (R 4.0.3 GUI 1.73 Catalina build (7892), using r studio (build 443).

3.3 Results

3.3.1 General performance

Epiphytic fouling was present on biomass in all three experiments. In the salinity experiment, fouling was present in all treatments; however, the low salinity treatments (5 and 15 ppt) had a higher level of fouling than the higher salinity treatments. In the exposure experiment, biomass in all exposure treatments had some level of fouling, however the 0- and 3-hour treatments had the least fouling and remained healthy in appearance throughout the experiment. Fouling in the 6- and 9-hour treatments increased over time. Similarly, in the light limitation experiment, all treatments sustained fouling throughout the experiment, however, the level of fouling increased over time in the 0% and 25% light limitation treatments but decreased over time in the 75% light limitation treatment and the pulse treatment. Additionally, all replicates of the 75% light limitation treatment were almost entirely free of all fouling by the final harvest.

Biomass bleaching was also an issue. In the exposure experiment there was consistent bleaching of the branchlet tips of biomass in both of the long exposure treatments (6- and 9-hours), while the 3-hour treatment experienced only minor bleaching in some replicates in the second week of the experiment. The extent of bleaching increased throughout the experiment in all three of the treatments that experienced exposure (3, 6, and 9 hours) however, patches of healthy biomass remained. In the light limitation experiment, there was some bleaching in the 0%, and 25% light limitation treatments. The biomass in the 75% light limitation treatment, however, did not experience any bleaching and darkened in colour to become dark brown by weeks two and three (Appendix 3.1). The biomass in the pulse treatment also remained healthy in appearance and darkened in colour. The biomass in the salinity experiment remained free of bleaching.

3.3.2 Salinity experiment

Specific growth rates (SGR) varied significantly between both salinity treatments and harvests (Table 3.1, Figure 3.2). SGR decreased with decreasing salinity and ranged from 5.0 % ± 0.3 S.E. in the 35 ppt treatment to 7.5 % ± 0.4 S.E. in the 5 ppt treatment. The 25 ppt and 15 ppt salinity treatments were 7% and 20% lower than the 35 ppt treatment respectively, and the 5 ppt treatment was 34% lower than the 35 ppt treatment. SGR across all salinity treatments was 32 – 44% higher in the first week of the experiment than over the following three weeks and ranged from 9.0 % ± 0.9 S.E. in week one to 5.0 % ± 0.3 S.E. in week three. FW:DW ratios ranged from 6.0 ± 0.1 S.E. to 4.2 ± 0.1 S.E. but did not vary consistently with salinity treatment or harvest, as indicated by a significant salinity x harvest interaction effect (Table 3.1, Figure 3.3)

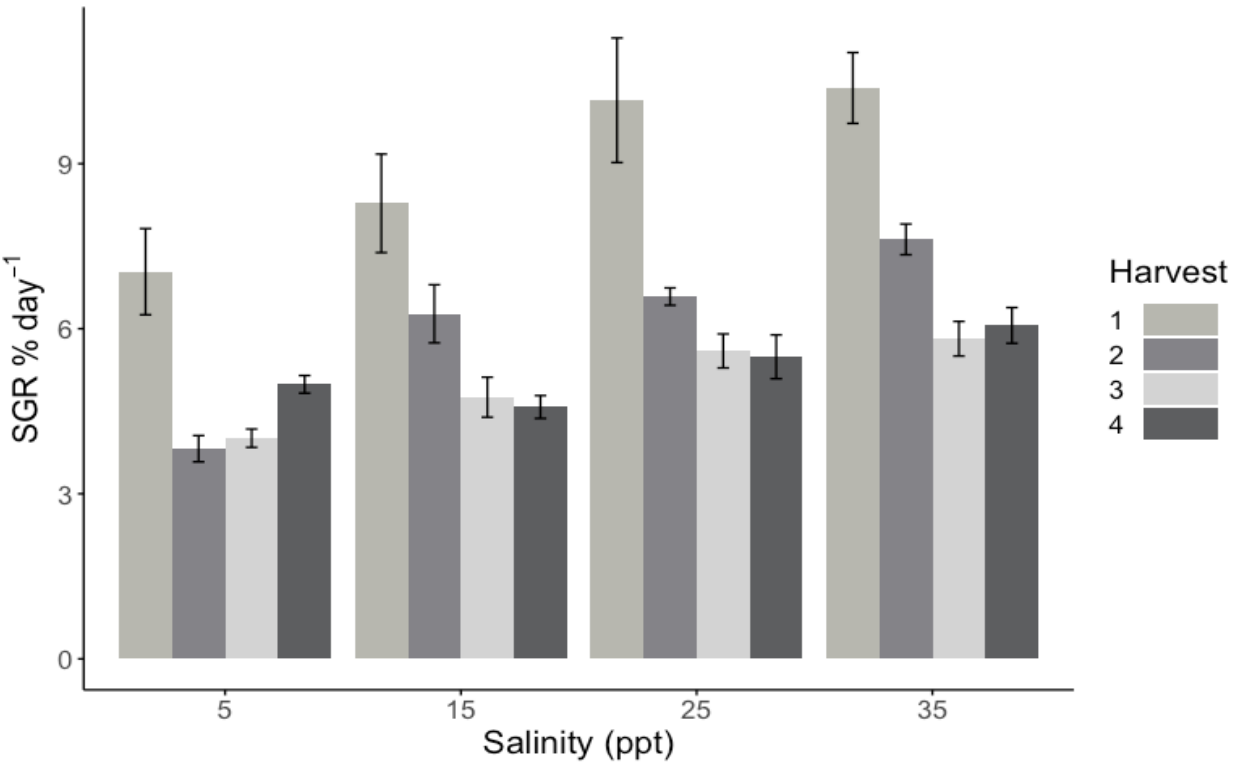

Figure 3.2: Mean (±SE) fresh weight to dry weight (FW:DW) ratios of *Gracilaria transtasmanica* biomass grown under four salinity treatments, over four weekly harvests.

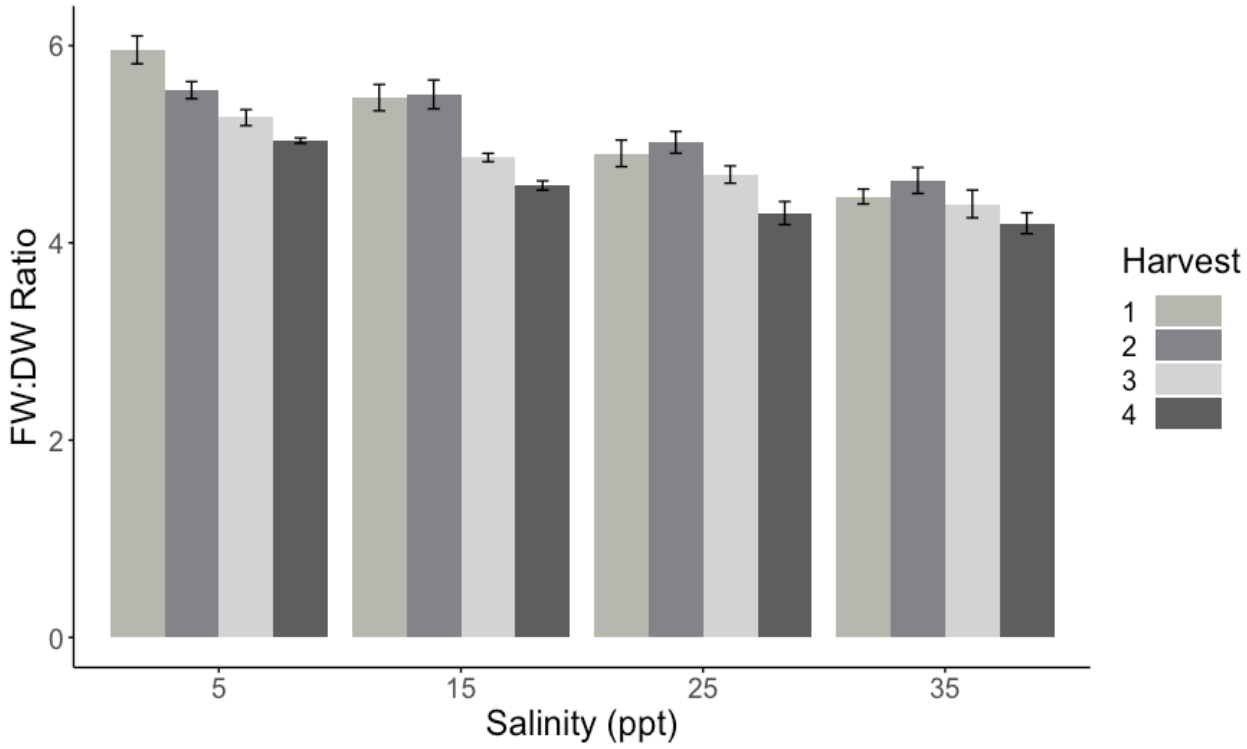

Figure 3.3: Mean (±SE) fresh weight to dry weight (FW:DW) ratios of Gracilaria transtasmanica biomass grown under four salinity treatments, over four weekly harvests.

Effective quantum yield ranged from 0.58 ± 0.02 S.E. to 0.61 ± 0.03 S.E. and was not significantly affected by either salinity treatment or day (Table 3.1, Figure 3.3). In contrast, optimal quantum yield varied significantly with day but not with salinity treatment (Table 3.1, Figure 3.3). Optimum quantum yield showed the highest variation in the first few days of the experiment, ranging from 0.56 ± 0.03 S.E. on days one and two to 0.44 ± 0.06 S.E. on day three across all salinity treatments, then showed little variation thereafter, ranging from 0.50 ± 0.03 S.E. on day six to 0.55 ± 0.04 S.E. on day 27.

Table 3.1: Results of analyses of variance (ANOVAs) testing the effects of salinity (Sal) and harvest (H) on Specific Growth Rate (SGR), and the effects and of salinity (Sal) and day (Da) on Effective quantum yield (Y(II)) and Optimal quantum yield (F_v/F_m) of *Gracilaria transtasmanica.* Degrees of freedom (df), F and P values are presented with significant P values in bold.

Factor	**Effect | Interaction**	**df**	**F**	**P**
SGR	Sal	3	18.567	**< 0.001**
	H	3	49.049	**< 0.001**
	Sal x Ha	9	1.648	0.121
	Res	64		
FW:DW	Sal	3	69.59	**< 0.001**
	H	3	35.91	**< 0.001**
	Sal x Ha	9	2.44	0.019
	Res	64		
Effective quantum yield (Y(II))	Sal	3	2.335	0.076
	Da	10	1.414	0.178
	Sal x Da	30	0.555	0.970
	Res	160		
Optimal quantum yield (F_v/F_m)	Sal	3	1.776	0.154
	Da	10	3.394	**< 0.001**
	Sal x Da	30	0.692	0.882
	Res	160		

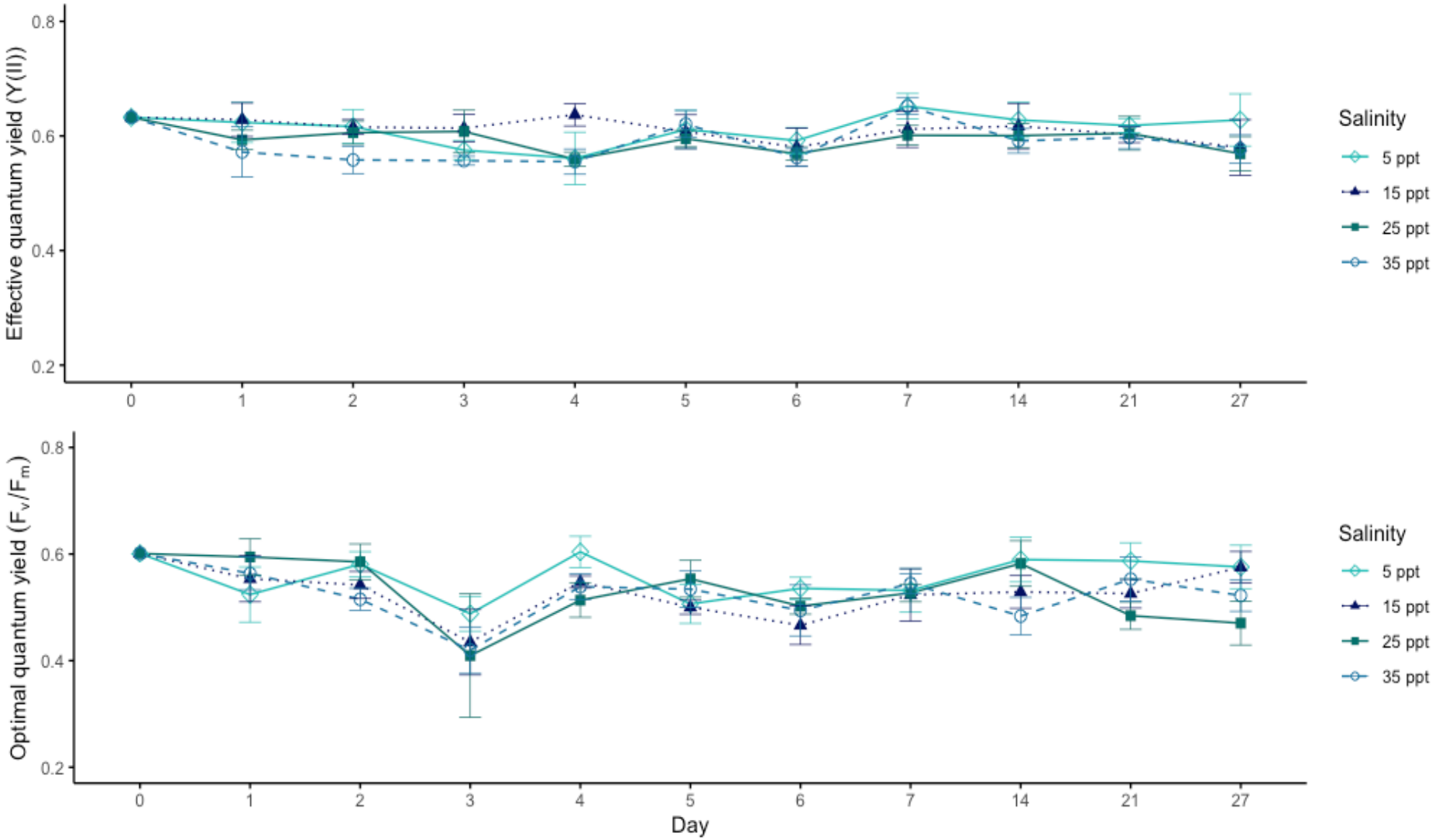

Figure 3.4: Mean (±SE) effective quantum yield (Y(II)), top panel) and optimal quantum yields (F_m/F_v, bottom panel) of *Gracilaria transtasmanica* grown under four salinity treatments over a four-week period.

3.3.3 Exposure experiment

Specific growth rates (SGR) varied significantly between exposure treatments, and decreased with increasing exposure (Table 3.2, Figure 3.5). Across all harvests, SGR was highest in the 0-hour exposure treatment (6.1 % ± 0.3 S.E.), and was 33%, 44% and 52% lower in the 3-hour (5.2 % ± 0.3 S.E.), 6-hour (4.3 % ± 0.7 S.E.), and 9-hour (2.9 % ± 0.6 S.E.) exposure treatments respectively. Harvest did not have a significant effect on growth (Table 3.3), with SGR ranging from 4.0 % ± 0.5 S.E. to 5.0 % ± 0.2 S.E. across all exposure treatments. FW:DW ratios were significantly different between exposure treatments but not between harvests (Table 3.2). Across all harvests, FW:DW ratios decreased from 4.3 ± 0.1 S.E. in the 9-hour exposure treatment to 3.9 ± 0.1 S.E. in the 0-hour exposure treatment (Figure 3.6).

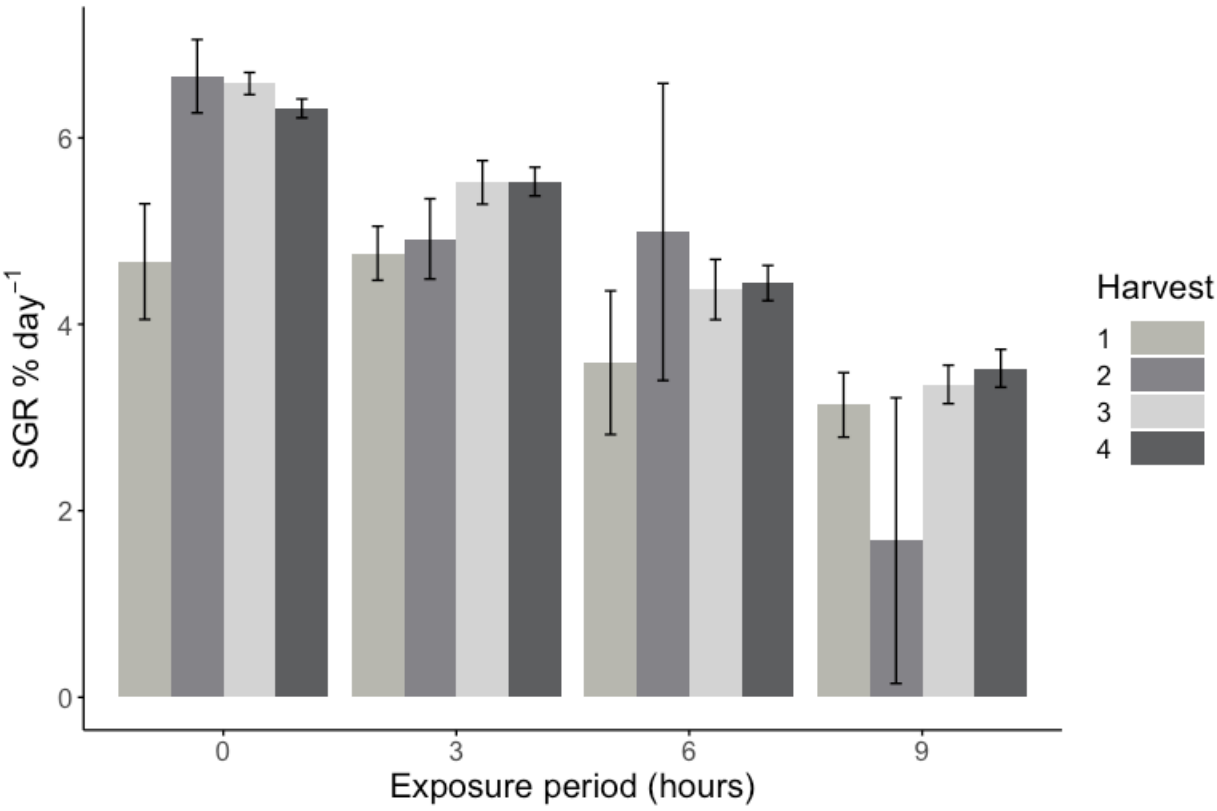

Figure 3.5: Mean (±SE) Specific Growth Rate (SGR, % day^{-1}) of *Gracilaria transtasmanica* biomass grown under four exposure treatments, over four weekly harvests.

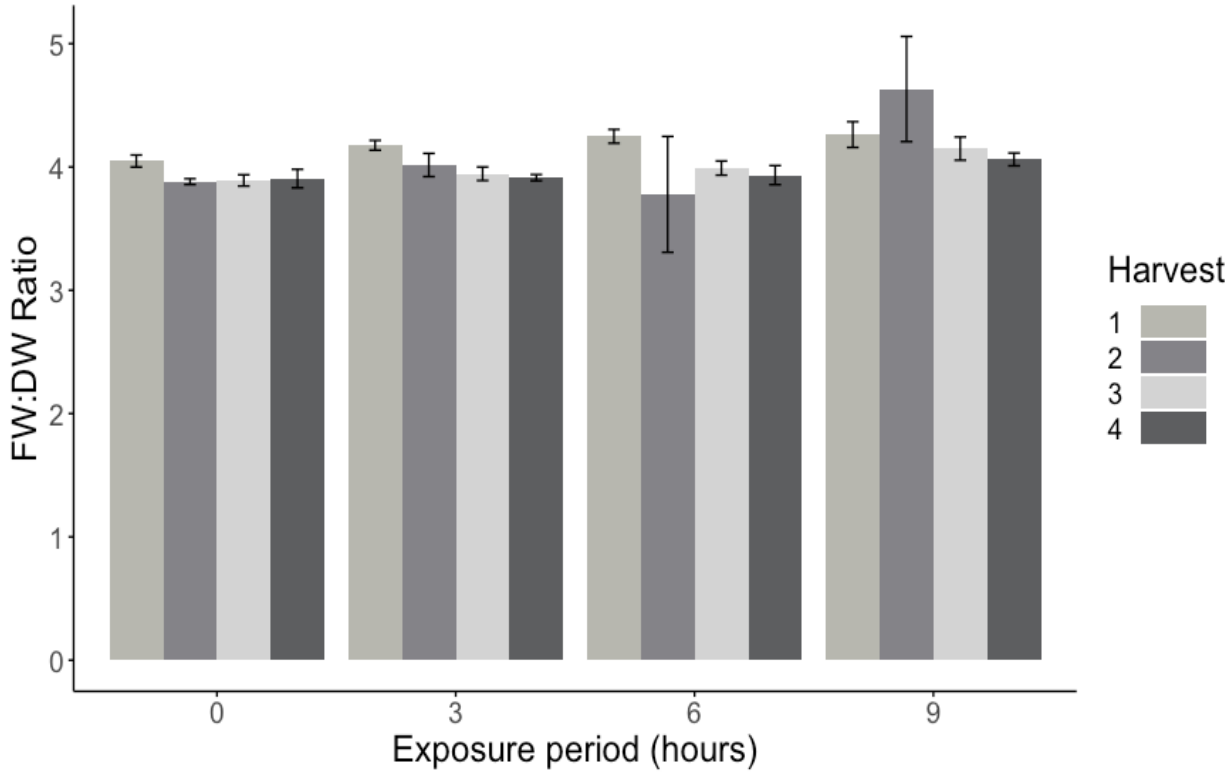

Figure 3.6: Mean (±SE) fresh weight to dry weight (FW:DW) ratios of *Gracilaria transtasmanica* biomass grown under four exposure treatments, over four weekly harvests.

Effective quantum yield did not vary significantly between exposure treatments but was significantly different between days, ranging from 0.63 ± 0.03 S.E. on day two to 0.44 ± 0.06 S.E. on day six across all exposure treatments (Table 3.2, Figure 3.7). Similarly, optimal quantum yield did not vary significantly with exposure treatment but was significantly different between days, ranging from 0.37 ± 0.05 S.E. on day six to 0.51 ± 0.03 S.E. on day 27 across all treatments (Table 3.2, Figure 3.7).

Table 3.2: Results of analyses of variance (ANOVAs) testing the effects of exposure (E) and harvest (H) on Specific Growth Rate (SGR), and the effects of exposure (E) and day (Da) on Effective quantum yield (Y(II)) and Optimal quantum yield (F_v/F_m) of *Gracilaria transtasmanica*. Degrees of freedom (df), F and P values are presented with significant P values in bold.

Factor	Effect | Interaction	df	F	P
SGR	E	3	16.883	**< 0.001**
	H	3	1.792	0.158
	E x Ha	9	1.053	0.410
	Res	64		
FW:DW	E	3	3.249	**0.028**
	H	3	1.431	0.242
	E x Ha	9	0.921	0.513
	Res	64		
Effective quantum yield (Y(II))	E	3	1.684	0.173
	Da	10	7.802	**< 0.001**
	E x Da	30	0.519	0.982
	Res	160		
Optimal quantum yield (F_v/F_m)	E	3	1.523	0.211
	Da	10	3.592	**< 0.001**
	E x Da	30	0.818	0.736
	Res	160		

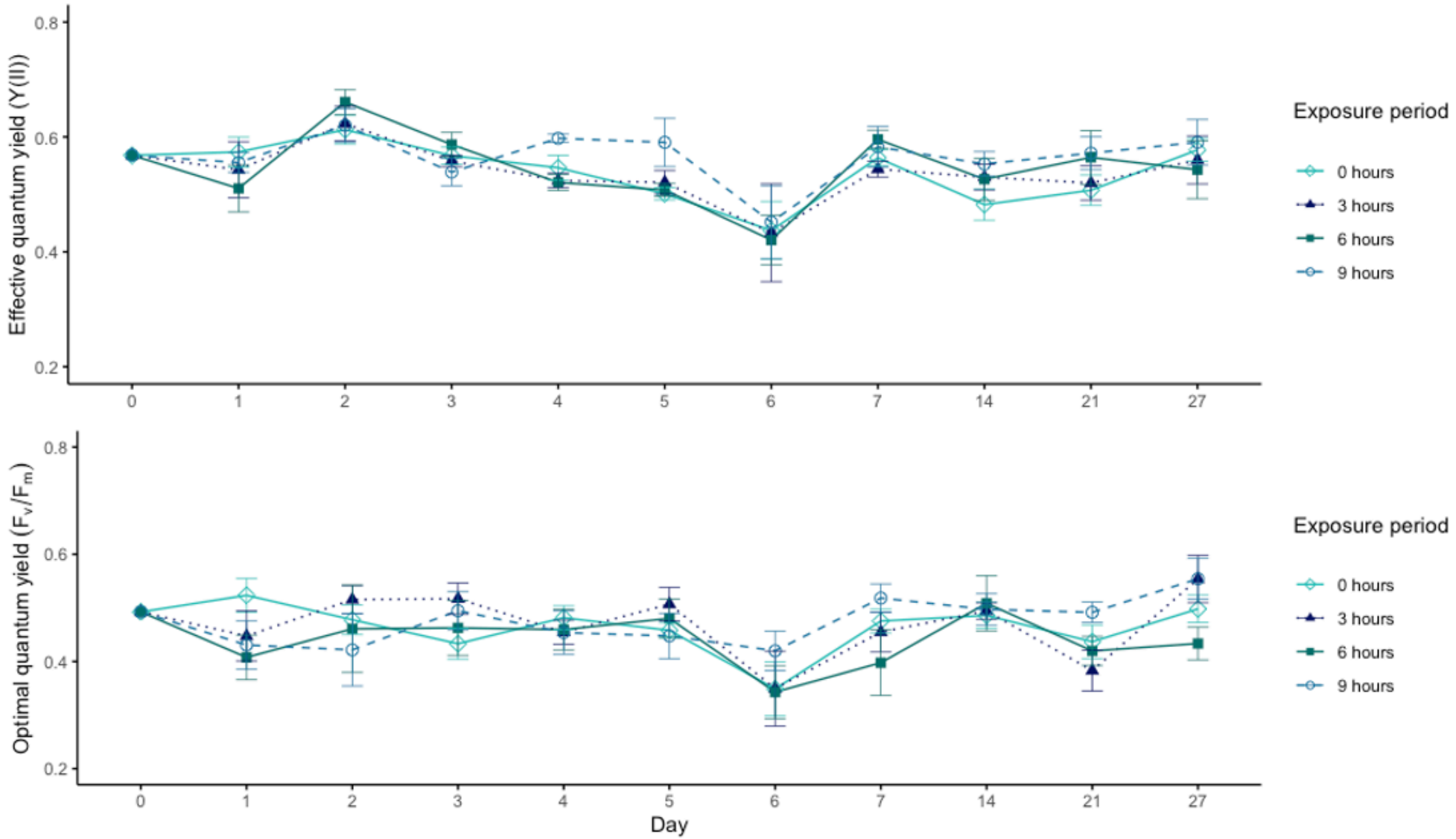

Figure 3.7: Mean (±SE) effective quantum yields (Y(II)), top panel); and optimal quantum yields (F_m/F_v, bottom panel) of *Gracilaria transtasmanica* biomass grown under four exposure periods over a four-week period.

3.3.4 Light limitation experiment

Specific growth rates (SGR) varied significantly between light limitation treatments and harvests (Table 3.3, Figure 3.8). Across all harvests, SGR was highest in the 0% light limitation treatment and decreased with increasing light limitation, ranging from 6.3 % ± 0.6 S.E. in the 0% light limitation treatment to 1.8 % ± 0.3 S.E. in the 75% light limitation treatment. SGR was 19% and 32% lower in the pulse and 25% light limitation treatments respectively compared to the 0% light limitation treatment and was 72% lower in the 75% light limitation treatment. Across all light limitation treatments, SGR increased by 20% between week one and week two (4.1 % ± 1.4 S.E. and 5.1 % ± 0.8 S.E. respectively) and thereafter decreased in week three and week four (4.4 ± 0.8 S.E. and 3.8 ± 0.8 S.E. respectively). FW:DW ratios were significantly affected by light limitation treatment and harvest (Table 3.3, Figure 3.9). Across all harvests FW:DW ratios were higher in the 75% and pulse light limitation treatments (4.1 ± 0.2, S.E. and 4.7 ± 0.2 S.E. respectively) than the 0% and 25% light limitation treatments (3.6 ± 0.2 S.E. and 3.9 ± 0.2 S.E.). Whilst across all light limitation treatments, FW:DW ratios ranged from 3.8 ± 0.2 S.E. in the first harvest to 4.3 ± 0.3 S.E. in the final harvest (Figure 3.9).

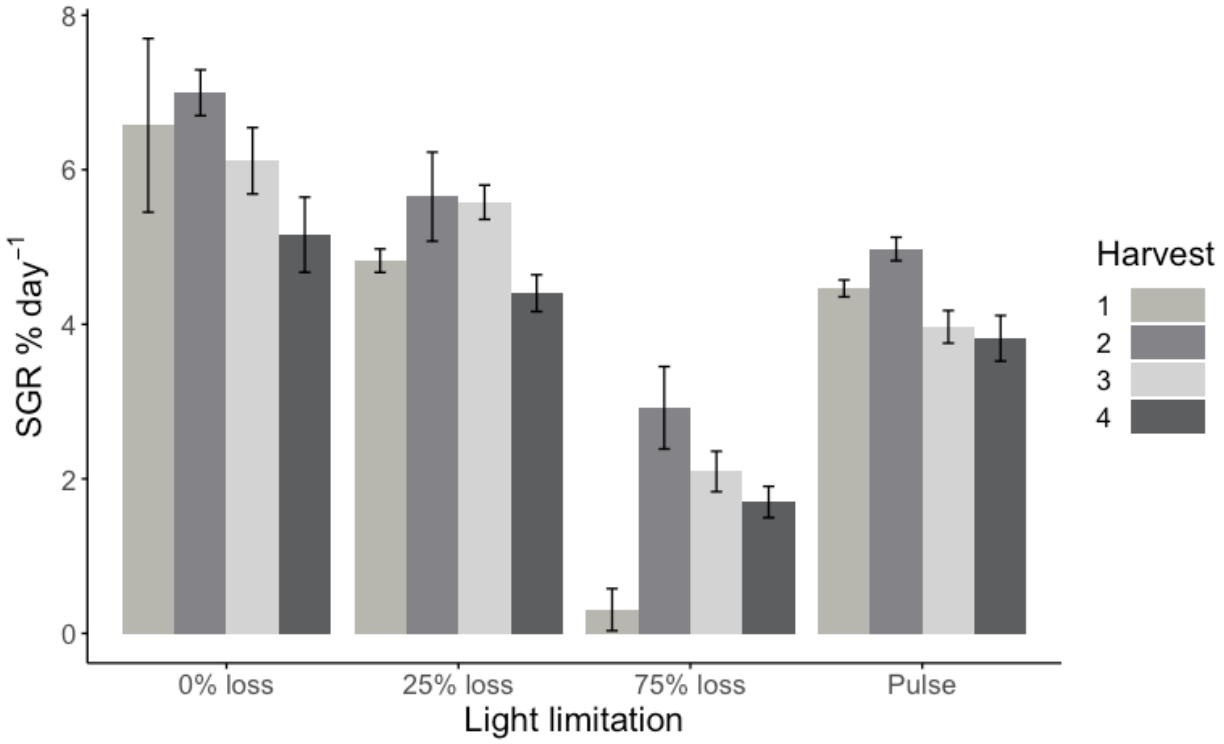

Figure 3.9: Mean (±SE) Specific Growth Rate (SGR, % day^{-1}) of *Gracilaria transtasmanica* biomass grown under four light limitation treatments over four weekly harvests.

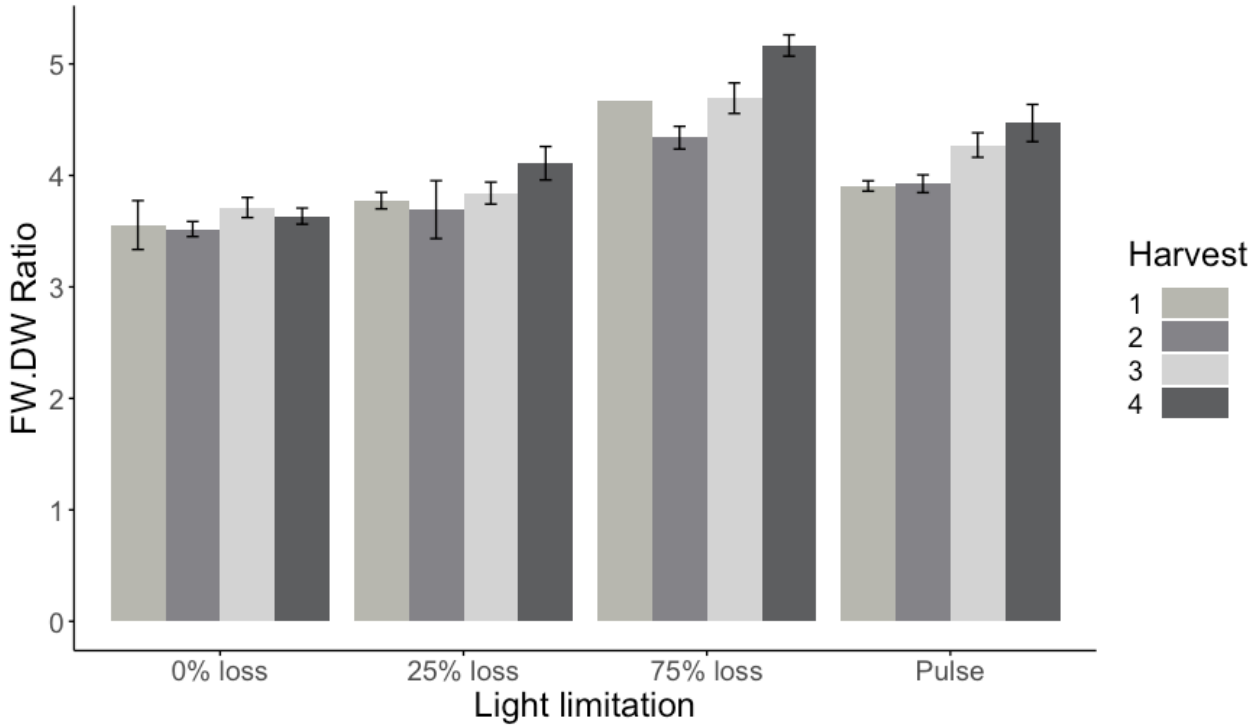

Figure 3.8: Mean (±SE) fresh weight to dry weight (FW:DW) ratios of *Gracilaria transtasmanica* biomass grown under four light limitation treatments, over four weekly harvests.

Both effective quantum yield and optimal quantum yield varied significantly among light limitation treatments, but this effect was not consistent among days, as indicated by significant light limitation x day interaction effects for both variables (Table 3.3, Figure 3.10). Across all treatments and days, effective quantum yield ranged from 0.55 ± 0.01 S.E. in the 0% light limitation treatment on day seven to 0.7 ± 0.01 S.E. in the 75% light limitation treatment on day four, and optimal quantum yield ranged from 0.46 ± 0.05 S.E. in the 0% light limitation treatment on the final day (27) to 0.65 ± 0.05 S.E. in the 75% light limitation treatment on day four.

Table 3.3: Results of analyses of variance (ANOVAs) testing the effects of light limitation (Li) and harvest (H) on Specific Growth Rate (SGR), and the effects of light limitation (Li) and day (Da) on Effective quantum yield (Y(II)) and Optimal quantum yield (F_v/F_m) of *Gracilaria transtasmanica*. Degrees of freedom (df), F value and P values are presented with significant P values in bold.

Factor	Effect | Interaction	df	F	P
SGR	Li	3	70.696	**< 0.001**
	Ha	3	6.431	**0.001**
	Li x Ha	9	1.910	0.066
	Res	64		
FW:DW	Li	3	45.096	**< 0.001**
	H	3	9.495	**< 0.001**
	Li x Ha	9	1.047	0.414
	Res	64		
Effective quantum yield (Y(II))	Li	3	1.972	0.120
	Da	10	7.014	**< 0.001**
	Li x Da	30	1.789	**0.012**
	Res	160		
Optimal quantum yield (F_v/F_m)	Li	3	2.975	**0.033**
	Da	10	3.316	**0.001**
	Li x Da	30	1.746	**0.015**
	Res	160		

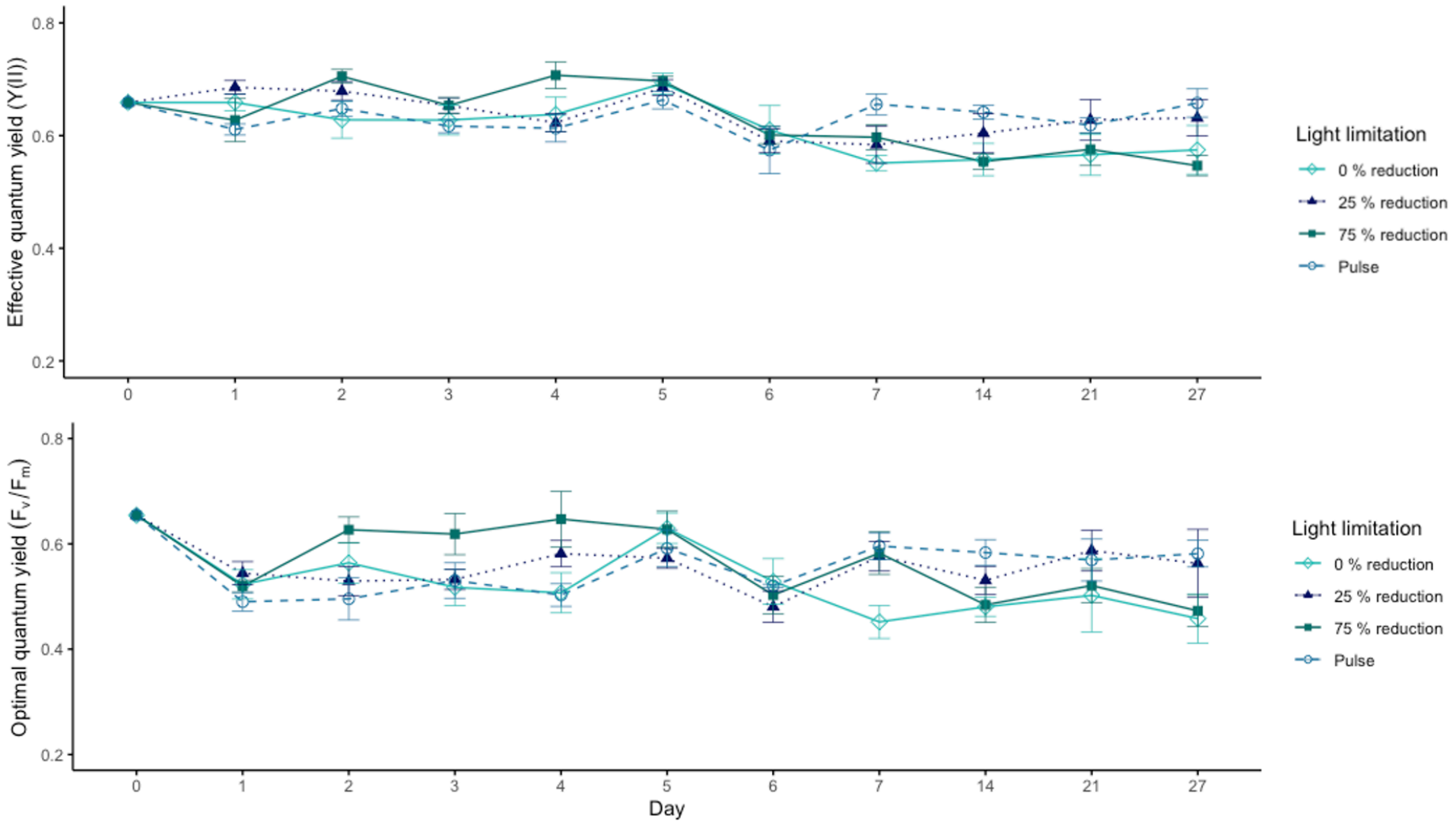

Figure 3.10: Mean (±SE) Effective quantum yields (Y(II)), top panel); and Optimal quantum yields (F_m/F_v, bottom panel) of *Gracilaria transtasmanica* biomass grown under four shade treatment levels inducing irradiance loss of zero, 25 %, 75 %, & blackout pulse, over a four-week period.

3.4 Discussion

A critical determinant of the suitability of macroalgal species for in-situ estuarine bioremediation is their ability to maintain productivity under a range of abiotic conditions (Abreu *et al.*, 2011; Lawton. *et al.*, 2013; Lawton *et al.*, 2014; Rose *et al.*, 2015; Roleda & Hurd, 2019). The controlled experiments conducted here demonstrated that *G. transtasmanica* can effectively maintain growth, and therefore productivity, under a broad range of abiotic conditions representative of ambient and extreme levels of salinity, exposure, and light limitation that typically occur in estuarine environments. Furthermore, fluctuations in these abiotic conditions did not generally have a negative effect on photosynthetic functioning and therefore biomass health. These results show that *G. transtasmanica* is robust in a range of challenging abiotic conditions.

The tolerance of macroalgae species to varying abiotic conditions can impact biomass growth (Dawes *et al.*, 1998; Neori *et al.*, 2004; Mata *et al.*, 2006; Barrington *et al.*, 2009; Abreu *et al.*, 2011; Karsten, 2012; Holzinger & Karsten, 2013; Scherner *et al.*, 2013). The current study demonstrated that *G. transtasmanica* is remarkably tolerant to low salinities, and was able to maintain reasonable growth rates in salinities as low as 5 ppt. This was expected following the observations of large quantities of healthy wild *G. transtasmanica* biomass growing in upper estuarine environments where salinities are low (Huanel *et al.*, 2020; Preuss *et al.*, 2020; Dudley *et al.*, 2022). Contrastingly, *G. transtasmanica* was not as tolerant to high periods of exposure (6- and 9- hours), reflected by lower growth rates recorded under these treatments. This poor growth was most likely a result of the biomass experiencing stress from desiccation and extreme irradiance in both of these treatments during periods of exposure (Dring *et al.*, 2001; Mata *et al.*, 2006). However, tolerance to increased exposure may be higher in winter when irradiance is

lower. The light limitation experiment showed that *G. transtasmanica* has a high tolerance for highly variable light levels, including low light and even short periods of no light. Although growth was highest in the treatment with no light limitation, the pulse light limitation treatment showed higher and more stable growth than the 75% light limitation treatment over time. Additionally, growth in the pulse treatment increased considerably (90%) from week one to week two, indicating strong acclimation to low light. The reasonably high and increasing growth rates in the pulse light limitation treatment shows that the biomass can tolerate and recover from limiting conditions, demonstrating that *G. transtasmanica* would be ideal for cultivation in estuaries where the biomass will experience short term extreme light limitation from sediment pulses caused by floods. Overall, these results reveal that *G. transtasmanica* grew best with limited exposure (0 – 3 hours), some light limitation (90 – 150 µmol photons m^{-2} s^{-1}), and in salinities from 15 – 35 ppt. Consequently, cultivation of *G. transtasmanica* is likely to be successful across a broad range of estuarine habitats, with highest growth rates expected in submerged subtidal channels and lower intertidal mudflats.

Abiotic stressors can have direct effects on cell health, and therefore tolerance and acclimation of photosynthetic functioning (Neori *et al.*, 2004; Mata *et al.*, 2006; Barrington *et al.*, 2009; Abreu *et al.*, 2011; Karsten, 2012; Scherner *et al.*, 2013). Measurements of effective and optimal quantum yields showed minimal effects of salinity treatment on the health and functioning of photosystem II. However, optimal quantum yield was negatively impacted in all treatments on day three of the salinity experiment, showing some long-term damage had occurred. This was likely linked to a technical error resulting in the lights on one half of the replicates remaining on for a full 24hr per day for the first three days of the experiment. However, the biomass recovered with optimal quantum yields showing a significant improvement in cell health within just

one day following the return to normal light settings (12:12 L:D). In the exposure experiment, measurements of effective and optimal quantum yield in the 6- and 9-hour exposure period treatments were comparable to the 0- and 3-hour exposure treatments and showed no long-term damage to photosynthetic health. While in the light limitation experiment, the 75% light limitation treatment had comparable effective and optimal quantum yields to the no light limitation and 25% light limitation treatments. Additionally, the 25% and pulse light limitation treatments showed improved and stable effective and optimal quantum yields in the final three weeks of the experiment. These results suggest that *G. transtasmanica* has some photosynthetic plasticity and can rapidly recover from and acclimate to short periods of both extreme irradiance and light limitation. The higher measurements of effective and optimal quantum yields following periods of low measurements in all three experiments show that *G. transtasmanica* recovered photosynthetic health and functioning following periods of stress. Therefore, although growth was negatively impacted by exposure and light limitation, these factors did not appear to have long-term effects on the performance of photosystem II. This result contrasts with findings reported for other species of red algae, which show that exposure and/or light limitation can have long term effects on photosynthetic functioning (Dawes *et al.*, 1998; Orduña-Rojas *et al.*, 2002; Mata *et al.*, 2006). Robustness and adaptability are critical traits for successful domestication and cultivation. The findings in the present study show *G. transtasmanica* to have similar adaptability to the commercially cultivated species *Gracilaria tikvahiae* and the invasive species *Gracilaria vermiculophylla*, which are both highly versatile and robust in estuarine environments (Yarish et al., 2012; Gorman et al., 2017; Preuss et al., 2020). Thus, *G. transtasmanica* is likely to be a dynamic and robust species for cultivation within estuarine environments.

Many seaweeds, including species in the genus *Gracilaria,* can alter tissue pigments under changing and challenging light conditions (Andria *et al.*, 1999; Pereira *et al.*, 2012). Under varied levels of light limitation in the current study, the biomass underwent notable changes in colour, most noticeably in the 75% light limitation treatment. This change in colour took place over a week during which growth was negligible, however, there was a considerable increase in growth thereafter. Additionally, the biomass in the 75% light limitation treatment had better photosynthetic health than the 0% light limitation treatments, as indicated by higher effective and optimal quantum yields (up to 20 and 30% respectively). This change in colour in the treatments with less light limitation could be explained by nutrient depletion or dissolved inorganic carbon (DIC) enrichment, as pigment loss has been found to result from these factors (García-Sánchez *et al.*, 1994; Andria *et al.*, 1999; Orduña-Rojas *et al.*, 2002). Further experimental work is required to refine understanding of the importance of nutrient and DIC dynamics in *G. transtasmanica* pigmentation, productivity, and acclimation.

Bioremediation potential is largely considered to be dependent on growth and productivity (Neori *et al.*, 2004; Barrington *et al.*, 2009; Abreu *et al.*, 2011; Scherner *et al.*, 2013), and under optimal conditions *G. transtasmanica* has been shown to dramatically increase both growth and N removal (Chapter 2). Exposure and extreme light limitation were the most limiting of the abiotic factors tested here. Biomass in the 3- to 9- hour exposure treatments had 33 – 52 % less growth than biomass that remained fully submerged. However, lower growth rates in the higher exposure treatments may also be linked to nutrient limitation as the biomass is unable to take up nutrients when exposed (Bracken, 2004). Nutrient limitation can also impact on growth strategy, acclimation ability and cell repair, and therefore, will have compounding effects if continued over time (Pedersen & Borum, 1997; Mata *et al.*, 2006; Martínez *et al.*, 2012;

Boderskov *et al.*, 2016). Extreme levels of light limitation also had a strong influence on growth, with 19 and 72 % less growth in the 25% and 75% light limitation treatments compared to the no light limitation treatment. However, work in Chapter 2 showed that N removal can be maintained under low light conditions when productivity is low. Therefore, although light limitation negatively impacted growth in the current study, bioremediation may not be similarly impacted. Salinity also impacted growth but is not considered to be a major threat to growth or bioremediation ability as differences in growth between salinity treatments were moderate at most (17 – 34% difference in SGR between low salinity treatments and the 35 ppt treatment). In combination, these results, suggest that tidal level (and therefore exposure duration) and proximity to freshwater inflows will be the most critical factors to consider when selecting specific locations within estuaries for culturing *G. transtasmanica*. As *G. transtasmanica* is affected by extended periods of light limitation and low salinity, locations closer to ocean outflows where the impacts of freshwater inflows and associated lower salinity and heightened turbidity are likely to be reduced would be preferable.

Differing abiotic conditions, particularly salinity, can alter biomass FW:DW ratios and therefore have implications for biomass applications (Ebeling & Jenkins, 1985; Cole *et al.*, 2015; Lawton. *et al.*, 2017; Lawton *et al.*, 2021a). Therefore, the effects of abiotic conditions on biomass quality are also important to consider when selecting target species (Gao & McKinley, 1994; Saldarriaga-Hernandez *et al.*, 2020). A high water content in the biomass, leading to a high FW:DW ratio, means a greater input will be required to obtain dry biomass for processing and further applications (Ebeling & Jenkins, 1985). Therefore, a high FW:DW ratio is generally considered undesirable for economical bioproduct production (Ebeling & Jenkins, 1985). Salinity has a direct effect on the osmotic balance in the biomass, as the cells will take in or lose salts via concentration

gradients, affecting the water content within the cells (Dawes *et al.*, 1998; Karsten, 2012; Holzinger & Karsten, 2013). Unsurprisingly, low salinities elevated water content in the biomass in the current study, and therefore, increased FW:DW ratios (Cohen & Fong, 2004; Karsten, 2012). Additionally, FW:DW ratios were higher in treatments with longer exposure periods and greater light limitation. However, when biomass was cultured fully submerged, and under higher salinity (25 – 35 ppt) and high light levels, FW:DW ratios were lower. Therefore, these conditions are not only optimal for biomass growth (as described above), but are also ideal for lower, more desirable FW:DW ratios. Consequently, selecting appropriate locations within estuaries for cultivation will not only affect growth and biomass health, but also biomass quality and processing requirements for further applications.

3.4.1 Conclusion

Gracilaria transtasmanica is tolerant to a broad range of exposures, light limitation, and salinities and therefore meets the requirements of environmental robustness and adaptability desirable in a target species for in-situ bioremediation (Lawton. *et al.*, 2013). Consequently, this species could be cultivated in a range of habitat types within estuaries. However, the optimal habitats for cultivation will be submerged subtidal channels and lower intertidal mudflats where the impacts of freshwater inflows and exposure are reduced. These same habitats will also produce optimal FW:DW ratios for biomass production. As the effects of salinity, exposure and light limitation were tested separately in the current study, future research should investigate whether there are interactive effects between these factors. In particular, whether there are compounding negative effects if the extremes of each factor (e.g., low salinity, low light and long periods of exposure) are experienced simultaneously. Furthermore, as noted in Chapter 2, seasonal

effects directly impact biomass growth and will therefore need to be considered in the selection of optimal habitats for cultivation. Seasonal changes in irradiance will be most important to consider as light extremes can damage biomass tissue and reduce growth and bioremediation efficiency, and these light extremes will differ across spatial scales and seasons. Finally, performance of *G. transtasmanica* should be quantified in-situ within estuarine habitats to verify the tolerances and effects of salinity, exposure and light limitation on growth identified here under controlled environmental conditions.

Chapter 4
General discussion

4.1 Thesis rationale

Estuaries provide invaluable ecosystem services to the human population (O'Higgins *et al.*, 2020), and are hubs for anthropogenic activities such as transport and shipping. They are therefore economically and socially important (Kennish, 2002; Flemer & Champ, 2006). However, estuaries both in New Zealand and globally are increasingly at risk of, or are succumbing to, eutrophication as a result of nutrient enrichment (Bricker *et al.*, 2008; Statham, 2012; Malone & Newton, 2020; Plew *et al.*, 2020). Eutrophication causes severe oxygen depletion, creating lethal conditions for organisms within the habitat, reducing species diversity, and therefore the resilience of the biological community (Thrush *et al.*, 2003; Thrush *et al.*, 2013; O'Meara *et al.*, 2017; Malone & Newton, 2020). Low diversity and resilience can negatively affect the provision of ecosystem services provided by estuaries which are crucial to ecology, society, economy (O'Higgins *et al.*, 2020). Furthermore, exposure to nutrient-enriched estuaries also increases the impacts of disease and pollution on human populations (Flemer & Champ, 2006). For these reasons, the protection and restoration of estuarine habitats are unquestionably both relevant and imperative (Flemer & Champ, 2006). Therefore, it is critical that a viable method of nutrient mitigation is developed to reduce and prevent estuarine eutrophication.

4.2 Key findings & bioremediation potential

The findings of the current study show that *G. transtasmanica* has considerable potential for in-situ bioremediation of nutrient enriched estuaries (Table 4.1). Experiments under ambient summer and winter conditions showed that *G. transtasmanica* can maintain nutrient removal year-round under background and elevated turbid conditions. Productivity was a strong predictor of nutrient removal in high nutrient and optimal light and temperature conditions. During periods of suboptimal winter conditions, *G. transtasmanica* productivity was low compared to summer conditions, however high rates of nitrogen removal were maintained. Additionally, when cultured in both low and high nutrient concentrations, *G. transtasmanica* proved very effective in taking up almost all available DRP. Controlled laboratory experiments showed that *G. transtasmanica* can maintain productivity under considerable fluctuation in salinity. Furthermore, this species shows plasticity in its ability to adapt to extreme light limitation. This demonstrates that *G. transtasmanica* is not only effective at bioremediation, but also robust to a wide range of abiotic conditions, a necessary characteristic for a species targeted for in-situ estuarine bioremediation (Lawton. *et al.*, 2013; Rose *et al.*, 2015).

Selection of cultivation sites to ensure the greatest impact in terms of nutrient removal will be critical for successful in-situ bioremediation. Assessments of *G. transtasmanica* tolerance to abiotic conditions showed reduced growth with higher low-tide exposure and extreme light limitation over extended periods of time. This indicates that selection of estuarine habitats such as sub-tidal channels will be preferable for optimal growth as the biomass will be constantly submerged. Locations or habitats that may receive extreme irradiance during summer months should be avoided. Additionally, as salinity tolerance was found to be relatively broad, locations of high nutrient input close to freshwater

inflows can be considered for cultivation, particularly as the light limitation treatment simulating a short turbidity flux occurring with a flood event at a river mouth did not have a large impact on growth. However, it should also be noted that productivity is not necessarily correlated with nutrient removal rate, due to the ability of *G. transtasmanica* to take up nitrogen independent of growth. Therefore, the biomass may still bioremediate effectively when growth is reduced in times of light limitation or extreme exposure. However, experiments measuring productivity and nutrient removal rates under summer and winter conditions were relatively short in duration (12 days). Therefore, nutrient uptake independent of productivity may diminish over time due to a reduced capacity for luxury uptake as nutrient stores become saturated.

Table 4.1: Summary of ash free dry weight (AFDW) productivity, nitrogen (N) removal, and elemental composition (% dry weight (DW)) of *Gracilaria transtasmanica* from experiments carried out in previous chapters. Environmental conditions are reported as optimal for growth, however, biomass will be able to maintain growth and N uptake outside of this range depending on various associated biological and abiotic factors. Data are means ± S.E. where appropriate. Note FAAs = Free Amino Acids, and ppt = parts per thousand.

Factor	**min**	**max**
Biomass Productivity (g AFDW m^{-2} day^{-1})	0.1 (± 0.1)	4.3 (± 0.1)
SGR (% day^{-1})	2.9 (± 1.8)	7.5 (± 2.1)
FW:DW Ratio	3.6 (± 0.3)	5.5 (± 0.4)
N Removal rate (mg N g DW biomass $growth^{-1}$)	12.4 (± 0.7)	35.4 (± 0.5)
Total N removal (g m^{-2} day^{-1})	5.4 (± 2.4)	33.4 (± 1.0)
Nitrogen %	1.4 (± 0.1)	4.1 (± 0.1)
Protein % (N conversion factor of 5)	7	20.5
FAAs – Gigartinine (µg g DW biomass $growth^{-1}$)	99.4 (± 20.1)	533.6 (± 30.3)
Ash (% DW)	22.3 (± 0.5)	27.8 (± 0.5)
Carbon (% DW)	29.9 (± 0.4)	33.7 (± 0.1)
Hydrogen (% DW)	4.7 (± 0.1)	5.9 (± 0.2)
Sulfur (% DW)	2.1 (± 0.1)	2.6 (± 0.0)
Salinity (ppt)	15	35
Exposure (hours)	0	3
Light limitation (µmol photons m^{-2} sec^{-1})	90	150

4.3 Barriers to implementation

In-situ macroalgal bioremediation could provide a solution to the on-going challenge of nutrient enrichment currently occurring in many New Zealand estuaries (Fei, 2004; Kim *et al.*, 2014, 2015; Seghetta *et al.*, 2016; Saldarriaga-Hernandez *et al.*, 2020; Plew *et al.*, 2020). As macroalgal bioremediation is a relatively new concept, there will be some barriers to implementation (Rose *et al.*, 2015; Neveux *et al.*, 2018). Cultivation of macroalgae in coastal environments requires in-situ infrastructure (Wood *et al.*, 2017; Thomas *et al.*, 2019). For example, cultivation lines will need mooring blocks and/or anchors embedded, the installation of which will cause some disturbance to the benthos (Wood *et al.*, 2017; Thomas *et al.*, 2019). The initial installment of these will have a short-term negative impact, furthermore, larger cultivation sites may alter hydrodynamics, and the amount of light that will reach the benthos, which could have ongoing negative impacts (Wood *et al.*, 2017). However, the long-term gains from the creation of 'new' habitats, as well as bioremediation services from macroalgae farms may be considered to more than offset these impacts (Theuerkauf *et al.*, 2022). There may also be cultural and environmental concerns around the translocation of domesticated cultivars of target species between sites and locations (Nepper-Davidsen *et al.*, 2021). These concerns can be addressed through the use of local cultivars collected where possible from each estuary where in-situ macroalgal bioremediation is to be implemented. Although nutrient removal is the primary product of in-situ bioremediation, the full potential of this approach can only be realized through appropriate use of the harvested biomass, thereby recycling the nutrients that the macroalgae has removed (Ferreira, 2017; Shahid *et al.*, 2020; Saldarriaga-Hernandez *et al.*, 2020). A key barrier to implementation is the current lack of commercially relevant biomass applications for macroalgae in exports or local industry (Rose *et al.*, 2015; Ferreira, 2017; Shahid *et al.*, 2020; Saldarriaga-Hernandez *et al.*,

2020). Therefore, assessing possible uses and markets for *G. transtasmanica* biomass is critical.

4.4 Circular economy potential

Macroalgal bioremediation enables sustainable industry opportunities through nutrient recovery and recycling, and the production of algal based bioproducts (Saldarriaga-Hernandez *et al.*, 2020; Shahid *et al.*, 2020). As of 2008, more than half of the synthetic fertilizer ever used was produced since 1985 (Howarth, 2008). This synthetic fertilizer has been heavily applied in agriculture, horticulture, and cropping (Howarth, 2008; Glibert, 2017; Malone & Newton, 2020). Consequently, as much as 80% of the nitrogen in protein within the human body is synthetically produced (Howarth, 2008). This introduction of 'new' synthetically produced nitrogen into the human diet and into the environment through human waste and agricultural runoff is the principal cause of coastal eutrophication (Howarth, 2008; Brown, 2019; Chakravarthy *et al.*, 2019; Plew *et al.*, 2020). Therefore, production of algal bioproducts such as animal feeds, biostimulants, and fertilizer, from macroalgal biomass cultivated in eutrophic waters can provide a pathway to recover and recycle these additional anthropogenic nutrients, thus avoiding the introduction of any more synthetically produced nutrients into the system (Figure 4.1) (Aitken & Senn, 1965; Lawton. *et al.*, 2017; Salcedo *et al.*, 2020). In addition, macroalgal biomass could be refined into carbohydrates and proteins for bioproducts that could further contribute directly to human diets, cosmetics, and health supplements (Figure 4.1) (Armisen, 1995; Maciel *et al.*, 2008; Tello-Ireland *et al.*, 2011; Fan *et al.*, 2012; Yarish *et al.*, 2012; Ferreira, 2017; Shahid *et al.*, 2020).

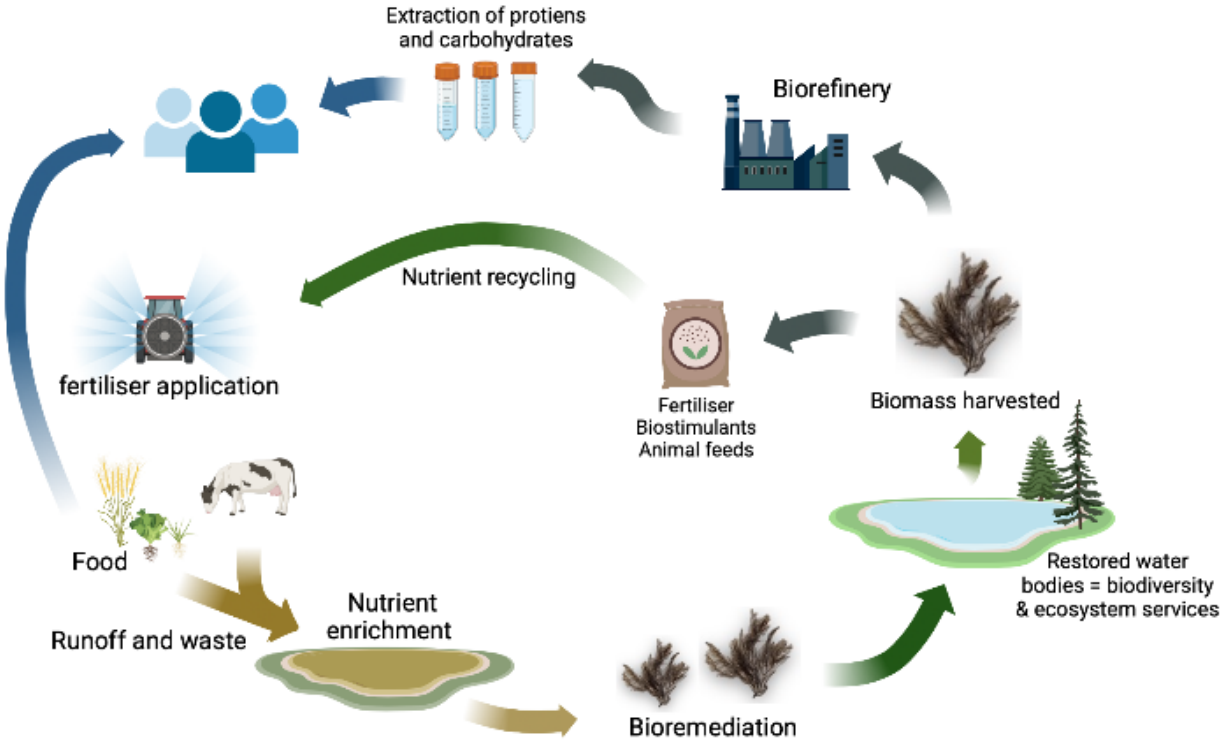

Figure 4.1: Schematic of *G. transtasmanica* bioremediation based circular bioeconomy. Created with biorender

4.5 Pathways and next steps: recommendations for further research

There is considerable potential to 'fast-track' implementation of *G. transtasmanica* cultivation by utilizing and adapting existing knowledge and aquaculture techniques for related species of *Gracilaria* that are commercially cultivated (Kain & Destombe, 1995; Yarish *et al.*, 2012; Rose *et al.*, 2015). However, further research and development is still required for successful implementation of in-situ macroalgal bioremediation with *G. transtasmanica* and optimized biomass performance and utilization. The results of this thesis highlight key areas for future research to enable the development of *G. transtasmanica* as a target species.

There are multiple factors that may impact optimal biomass productivity. High density can increase self-shading, and consequently could reduce impacts from extreme

irradiance or conversely limit productivity in low light (Carpenter, 1990; Mata *et al.*, 2006; Higgins *et al.*, 2008). Therefore, as *G. transtasmanica* can be negatively affected by high irradiance, and productivity can fluctuate with density, the interactions between density and irradiance, and their effects on productivity and nutrient removal should be investigated. In addition, as gigartinine production and biomass productivity require increased carbon molecules (Laycock & Craigie, 1977; PubChem, 2005), nutrient and carbon concentrations for optimal growth should be determined (Andria *et al.*, 1999) for *G. transtasmanica.* Pesticides used in horticulture and cropping, as well as emerging organic contaminants (EOCs) are frequently delivered to estuarine habitats (Chung *et al.*, 2011; Barletta *et al.*, 2019). Macroalgae are highly adept at taking up these contaminants into their tissue, thus actively remediating pollutants, however these contaminants may negatively impact growth and affect biomass applications (Neveux *et al.*, 2018; Saldarriaga-Hernandez *et al.*, 2020; Michalak, 2020). Therefore, the presence and potential impacts of herbicides, pesticides and EOCs on biomass growth, bioremediation, and biomass quality should be assessed (Chung *et al.*, 2011; Neveux *et al.*, 2018; Barletta *et al.*, 2019; Saldarriaga-Hernandez *et al.*, 2020).

Fouling was an on-going and fluctuating issue throughout this study. High levels of fouling can make it difficult to maintain healthy monocultures and can impact negatively on biomass applications (Phillips, 1990; Bannister *et al.*, 2019). Therefore, work is needed to explore causes and solutions. However, fouling was reduced under long periods of exposure and high (75%) light limitation, and therefore could be controlled through cultivation depth (increasing light limitation) and biomass density (inducing self-shading) (Mata *et al.*, 2006; Deng *et al.*, 2012; Liao *et al.*, 2018). Field observations showed that wild stocks of *G. transtasmanica* appear to be free of fouling. This may be linked with the presence of epibionts, often crustaceans living in and on the biomass, potentially

feeding on the epiphytic fouling species (Stachowicz & Hay, 1996; da Gama *et al.*, 2008; Newcombe & Taylor, 2010; Liao *et al.*, 2018). However, the lack of fouling in wild stocks of *G. transtasmanica* may also be a result of environmental factors not yet fully understood. Therefore, cultivation in estuarine environments, rather than in controlled batch cultures as in the present study, may result in a natural reduction in fouling. A further consideration could be co-culture with other species of seaweed (Wang *et al.*, 2009; Kang *et al.*, 2021). This study showed that *G. transtasmanica* meets the base requirements for a target bioremediation species. However, poor performance in low N conditions in summer and possible susceptibility to bleaching is a concern, and high levels of fouling in summer were also an issue. Therefore *G. transtasmanica* could be co-cultured with a summer-dominant species, such as *Ulva sp.,* presenting an opportunity to diversify and produce increased year-round results in terms of both bioremediation and productivity (Wang *et al.*, 2009; Rose *et al.*, 2015; Kang *et al.*, 2021).

Considering the presence of nitrogen-rich gigartinine in *G. transtasmanica,* potential uses as a source of protein-rich biomass should be explored. In particular, the content of all FAAs should be determined as interest in alternative protein sources has a growing focus on seaweeds (Černá, 2011; Bleakley & Hayes, 2017). Moreover, several *Gracilaria* species are cultured and harvested for polysaccharides such as agar, and demand for these products is increasing (Chapman & Chapman, 1980; Armisen, 1995; Maciel *et al.*, 2008; Tello-Ireland *et al.*, 2011; Yarish *et al.*, 2012; Fan *et al.*, 2012; Mantri *et al.*, 2020). Therefore, in addition to proteins, further analysis of the constituents of *G. transtasmanica* biomass and the effects of abiotic factors on their concentrations would enable a greater understanding of potential use of biomass cultivated in estuarine environments.

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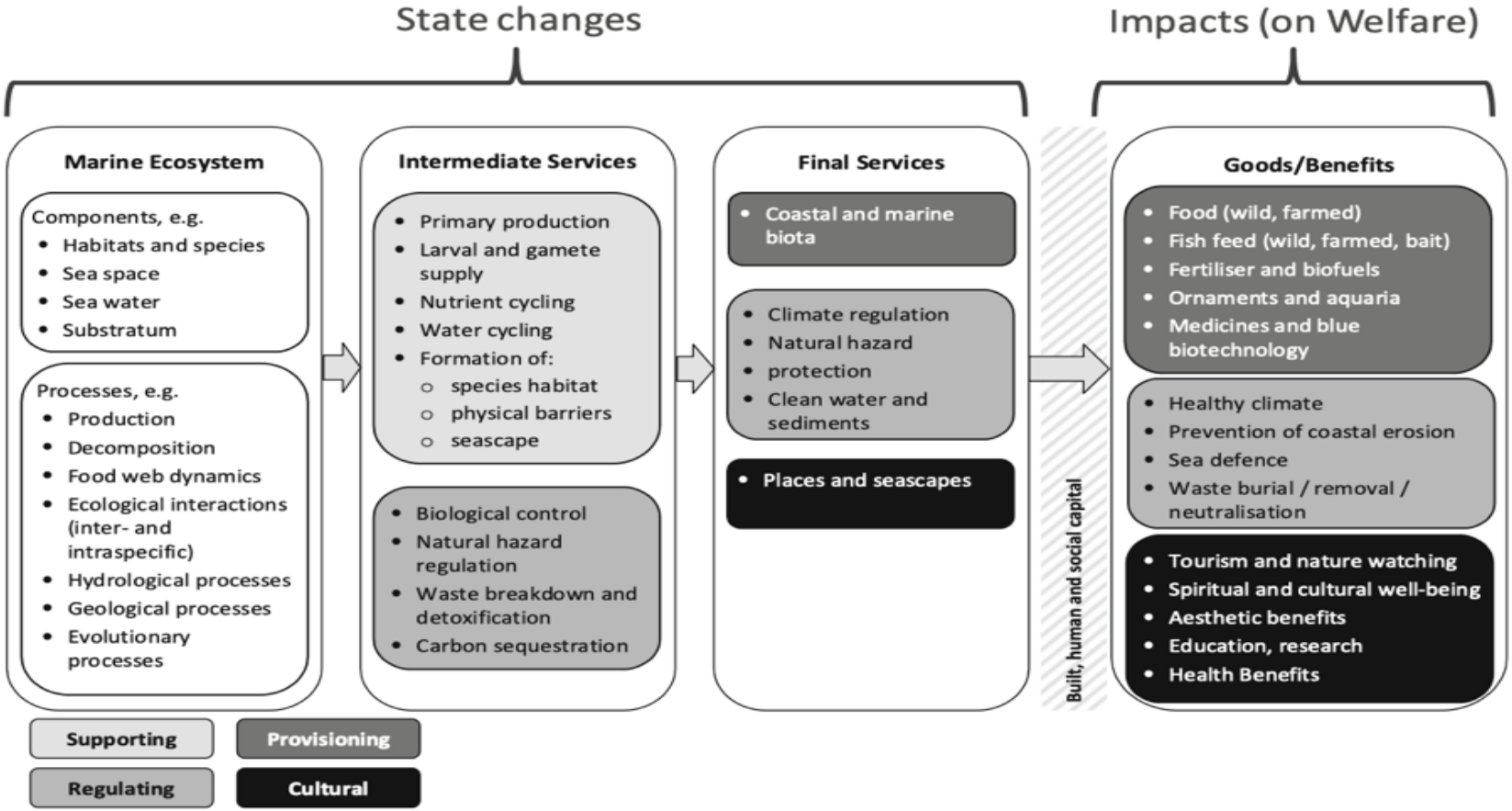
Appendices

Appendix 1.1.

Condition changes within the natural environment reflected by shifts in the marine ecosystem (including estuarine space), intermediate and final ecosystem services, impacts on human welfare, and changes to the provision of goods and services (ecosystem services) to the community. Sourced from (O'Higgins *et al.*, 2020).



Appendix 2.1.

Collection location and habitat, and morphological characteristics of samples of *Gracilaria transtasmanica* and *Gracilaria chilensis* collected for this study from three locations in Maketu and Little Waihi estuaries in the Bay of Plenty, New Zealand.

Sample	Species	Location	Coordinates		Collection habitat	Morphological characteristics
			Latitude	Longitude		
K-1	*G. transtasmanica*	Kaituna cut	37° 45' 34.998" S	176° 27' 57" E	Upper intertidal, silty/clay sediment, very exposed, dry	Thallus < 1mm. Lots of branchlets, hooked tips
K-2	*G. transtasmanica*	Kaituna cut	37° 45' 34.998" S	176° 27' 57" E	Fuzzy shorter growth. Anoxic sediment, silty/clay. Upper intertidal	Possible 5th order branchlets. Thallus < 1mm
K-3	*G. transtasmanica*	Kaituna cut	37° 45' 36" S	176° 27' 0" E	Large patch (5m^2) edge of channel, partly submerged	Thallus < 1mm. Branching around axis, four orders. Hooked tips
K-4	*G. transtasmanica*	Kaituna cut	37° 45' 7.998" S	176° 25' 1.002" E	Well defined individual, side of smaller channel, intertidal, plant slightly submerged	Thallus 1mm. Some branching. Length over 20cm
K-5	*G. transtasmanica*	Kaituna cut	37° 45' 36" S	176° 27' 0" E	Side of channel as above, mixed sediment, firmer ground	Thallus and branches > 1mm) lots of small branching.
K-6	*G. transtasmanica*	Kaituna cut	37° 44' 58.998" S	176° 25' 4.002" E	Side of third channel. Mixed sediment, low intertidal, submerged	Thallus < 1mm. Paler colour (more brown than black). No visible hooks on tips
M-1	*G. chilensis*	Maketu Estuary	37° 45' 43.998" S	176° 26' 43.002" E	Exposed mud flat area near shore, sample found in shallow pool	Thallus not clear, very "shrubby" many branches around the holdfast
*M-2	*G. chilensis?**	Maketu Estuary	37° 45' 43.998" S	176° 26' 43.002" E	Exposed mud flat area near shore, sample found in shallow pool	Fouling. Thallus > 1mm. Sparse branching
M-3	*G. transtasmanica*	Maketu Estuary	37° 45' 45" S	176° 26' 42" E	Single individual upper intertidal, exposed mudflat	Thallus 1mm. Lots of branches, slightly hooked tips,

						entire specimen longer than 25cm
M-4	*G. transtasmanica*	Maketu Estuary	37° 45' 40.998" S	176° 26' 49.998" E	Single individual upper intertidal, exposed mudflat	Thallus < 1mm, tips almost hooked
M-5	*G. transtasmanica*	Maketu Estuary	37° 45' 42" S	176° 26' 46.002" E	Small channel in the middle of estuary, mixed assemblage of algae at least 4 species, all reds. Sandy and silty, fouling from filaments	Thallus < 1mm. Four, possible five orders (branchlets). Hooked tips
M-6	*G. transtasmanica*	Maketu Estuary	37° 45' 36" S	176° 27' 0" E	As above	Thallus > 1mm. Length over 20cm. Third order branching, not hooked
M-7	*G. chilensis*	Maketu Estuary	37° 45' 31.998" S	176° 26' 46.998" E	Small channel, mid estuary, sandy	Thallus > 1mm. No hooks, thick lower branching, sparse higher branching.
M-8	*G. chilensis*	Maketu Estuary	37° 45' 37.002" S	176° 28' 42" E	Small channel, mid estuary, sandy	Thallus > 1mm. No hooks, thick lower branching, sparse higher branching, strong holdfast.
W-1	*G. transtasmanica*	Little Waihi	37° 45' 34.998" S	176° 28' 34.998" E	Silty clay and exposed, very high intertidal	Thallus < 1mm, fine
W-2	*G. transtasmanica*	Little Waihi	37° 45' 34.998" S	176° 28' 34.998" E	Silty clay, Exposed, intertidal	Slightly hooked tips (Thallus 1mm), fine
W-3	*G. chilensis*	Little Waihi	37° 45' 37.998" S	176° 28' 40.998" E	As above moving toward mid tidal plane	Thallus > 1mm, thick, and similar in appearance to M7 and M8
W-4	*G. chilensis*	Little Waihi	37° 45' 40.998" S	176° 28' 40.002" E	As above moving toward mid tidal plane	Thallus > 1mm, thick, and similar in appearance to M7 and M8
W-5	*G. chilensis*	Little Waihi	37° 45' 40.998" S	176° 28' 40.002" E	As above moving toward mid tidal plane	Thallus > 1mm, branching mostly at the base. No hooks

W-6	*G. transtasmanica*	Little Waihi	37° 45' 42" S	176° 28' 39" E	As above moving toward mid tidal plane	Thallus < 1mm, finer branching particularly towards the tips. Subtle hooked tips
W-7	*G. chilensis*	Little Waihi	37° 45' 42" S	176° 28' 39" E	Submerged in channel at low tide, covered in cystocarps	Thallus > 1mm, thick branches, similar again to M7/8
W-8	*G. chilensis*	Little Waihi	37° 76'12"S	176° 47'77"E	Submerged in channel at low tide, covered in cystocarps	Thallus > 1mm
W-9	*G. chilensis*	Little Waihi	37° 76'16"S	176° 47'74"E	Submerged in channel at low tide, covered in cystocarps	Thallus > 1mm
*Not included in genetic analyses due to extreme fouling, poor overall health in captivity. Thought to be *G. chilensis* based on morphological characteristics.						

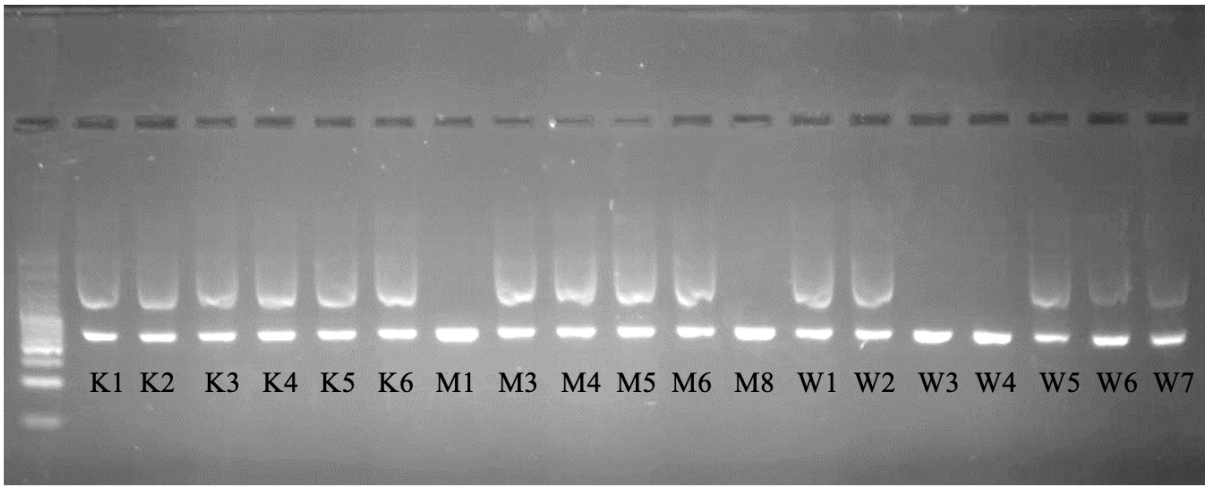
Appendix 2.2.

Udden-wentworth scale (Wentworth, 1922) of sediment size grade and class. From Blair and McPherson (1999)

PARTICLE LENGTH (dI) km	m	mm	ϕ	GRADE	CLASS	FRACTION Unlithified	FRACTION Lithified
1075			-30	very coarse	Megalith	Megagravel	Mega-conglomerate
538			-29	coarse			
269			-28	medium			
134			-27	fine			
67.2			-26	very fine			
33.6			-25	very coarse	Monolith		
16.8			-24	coarse			
8.4			-23	medium			
4.2			-22	fine			
2.1			-21	very fine			
1.0	1048.6		-20	very coarse	Slab		
0.5	524.3		-19	coarse			
0.26	262.1		-18	medium			
	131.1		-17	fine			
	65.5		-16	very coarse	Block		
	32.8		-15	coarse			
	16.4		-14	medium			
	8.2		-13	fine			
	4.1	4096	-12	very coarse	Boulder	Gravel	Conglomerate
	2.0	2048	-11	coarse			
	1.0	1024	-10	medium			
	0.5	512	-9	fine			
	0.25	256	-8	coarse	Cobble		
		128	-7	fine			
		64	-6	very coarse	Pebble		
		32	-5	coarse			
		16	-4	medium			
		8	-3	fine			
		4	-2		Granule		
		2	-1	very coarse	Sand	Sand	Sandstone
		1	0	coarse			
		0.50	1	medium			
		0.25	2	fine			
		0.125	3	very fine			
		0.063	4	coarse	Silt	Mud	Mudstone or Shale
		0.031	5	medium			
		0.015	6	fine			
		0.008	7	very fine			
		0.004	8		Clay ↓ ?		
		0.002	9				
		0.001	10				
		0.0005	11				
		0.0002	12				
		0.0001	13				

Appendix 2.3.

Agarose gel showing results of PCR diagnostic tool used to identify samples as *G. transtasmanica* (2 bands visible) and *G. chilensis* (1 band visible). Note W5 was re-tested as morphology notes did not match initial gentic anylses shown here, the repeated test confirmed this sample as *G. transtasmanica* not *G. chilensis*.



Appendix 2.4.

Photographs of samples showing morphological differences between *G. chilensis* (left) and *G. transtasmanica* (right). Note the overall much smaller finer appearance, thinner thallus and finer more branched filaments and the slight curved or "hooked" tips *in G. transtasmanica* compared to *G. chilensis*.

Appendix 3.1.

Image showing difference in biomass colouring following cultivation for 3 weeks under varied levels of light limitation in experiment three.