New Perspectives in Multi-trophic Aquaculture

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ABSTRACT

Aquaculture, the farming of marine and aquatic organisms, is currently the fastest growing food production sector globally. As capture fisheries have stagnated and the fishing down of marine food webs has been seen to be unsustainable, aquaculture is filling the deficit in providing food to a growing world population. Aquaculture species can be divided into two groups: those that require an external input of a food source, known as 'fed species', and those that extract their food and nutrients directly from the marine environment, known as 'non-fed' or 'extractive' species. Fed species are generally fish and shrimps, while extractive species are predominantly seaweeds and bivalves.

While seaweeds and bivalves are good candidates for aquaculture due to their nutritional profiles, compounds and wide array of applications, they are also promising from an environmental perspective. Filter feeding bivalves provide an uptake of particulate nitrogen while seaweeds sequester both dissolved nitrogen and carbon as well as providing an oxygenation effect. Thus, by absorbing carbon and nitrogen, extractive species have a positive environmental benefit as well as providing a valuable aquaculture yield. While the cultivation of other species, such as finfish, has been seen to cause environmental degradation, the cultivation of extractive species can be viewed as truly sustainable if farmed appropriately.

Integrated multi-trophic aquaculture (IMTA) is a method of aquaculture that incorporates multiple species into one farm. The aim is to prevent waste nutrients of one species being lost into the marine environment, but rather being captured and used by other species in the system, thus preventing environmental damage while producing an enhanced and more diverse crop. To date, IMTA systems have mostly centred around a fed species around which extractive species are placed. The aim of this thesis is to explore the potential for IMTA system containing only extractive species where no external inputs are necessary. Further, this thesis also explores the role of nutrient enrichment on the response of seaweed to environmental stress, as well as to explore the potential for multi-species cultivations of seaweed.

My findings show that by cultivating kelp in association with bivalves, kelp yields increase in terms of both quality and quantity, where kelp grow larger, contain more valuable bioactive compounds and display reduced rates of biofouling. By enhancing growth, the bioremediation capacity of seaweeds is also increased, thus increasing the total assimilated carbon and nitrogen. Further, I also show that the impacts of mussels on kelp are positive even in high nitrogen environments, such as around fish cultivations, resulting in enhancements of bioactive compounds in kelp tissue. Lastly, I report that the cultivation of multiple seaweed species together may be problematic due to nutrient competition and chemical interactions, although nutrient limitation may be mediated by nitrogen enrichment. Overall, this thesis presents new perspectives in multi-trophic aquaculture, where extractive species have benefits beyond bioremediation, and the potential for IMTA systems containing only extractive species is revealed.

Keywords: aquaculture, kelp, seaweed, IMTA, mussel, bioremediation, *Saccharina latissima, Mytilus edulis,* extractive aquaculture, biofouling, epiphytes

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vattenbruk är globalt sett den snabbaste livsmedelssektorn och är betydande för världsbefolkningen. Medan det globala fisket saktar ner och blir mindre produktivt, tillhandahåller vattenbruk livsmedel för en växande global befolkning. Vissa vattenbruksarter, såsom fisk, behöver en extern födokälla men andra arter som tång och musslor, så kallade extraktiva arter, fångar all näring från havet, där föda förekommer naturligt.

De flesta vattenbruk fokuserar på en art och vanligtvis fisk, såsom lax. Det finns dock en annan metod, där flera arter kan samodlas i ett och samma system. Dessa system använder både fisk och extraktiva arter, där näringsämnen som produceras av en art kan fångas upp och användas av en den andra. Detta innebär att extra näringsämnen från odlingar kan fångas upp innan de kan skada den marina miljön samt öka tillväxten hos de arter som använder näringsämnena.

Min doktorsavhandling testar utvecklingsmöjligheter inom vattenbruk genom att samodla flera arter i samma system där föda och näringsämnen inte behöver tillföras. Målet med detta är att skapa ett hållbart vattenbruk som tillhandahåller en rad ekosystemtjänster, såsom livsmedel, vattenrening genom filtrerande organismer samt lagring av näring och kol i vegetation och organismer.

Mina resultat visar att sockertång har större tillväxt, innehåller fler värdefulla föreningar och har färre påväxtorganismer (renare blad), när de samodlas med blåmusslor. Dessa resultat beror på att musslorna filtrerar partiklar i vattnet vilket förbättrar siktdjupet nedströms och i sin tur ökar ljusintensiteten under havsytan. Sockertången drar nytta av extra ljus och växer därmed snabbare. Att sockertången blir renare beror på att musslorna äter larver av andra organismer och avlägsnar därmed organismer som kan fästa sig på tången. Sammantaget innebär detta att skörden för tångodlingar ökar samtidigt som tången tar upp och lagrar mer kol och kväve, vilket gynnar den lokala miljön.

Sammanfattningsvis har min forskning visat att samodlingar inte behöver en utfodrad art för att fungera och att kombinera extraktiva arter bidrar till många ekosystemtjänster. Vidare kan resultaten från mina studier tillämpas längs den svenska västkusten där blåmussla är en typisk vattenbruksart och algodlingar blir allt mer populära.

LIST OF PAPERS

This thesis is based upon the following papers, referred to in the text by their roman numerals:

PAPER I: **Hargrave, M. S.**, Ekelund, A., Nylund G. M., Pavia. H. Filtration and fertilisation effects of the bivalves *Mytilus edulis* and *Magallana gigas* on the kelp *Saccharina latissima* in tank culture. *Submitted*

PAPER II: **Hargrave, M. S.**, Nylund G. M., Pavia. H. Co-cultivation with blue mussels increases yield and biomass quality of kelp. *Submitted*

PAPER III: Rugiu, L., **Hargrave, M. S.**, Enge, S., Sterner, M., Nylund, G. M., Pavia, H. (2020). Kelp in IMTAs: small variations in inorganic nitrogen concentrations drive different physiological responses of *Saccharina latissima. Journal of Applied Phycology,* 1-14.

PAPER IV: **Hargrave, M. S.**, Nylund G. M., Pavia. Nitrogen enrichment reduces the negative impacts of hyposalinity stress in *Saccharina latissima. Manuscript*

PAPER V: Rugiu, L., **Hargrave, M. S.**, Durland, E., Toth, G., Nylund, G. M., Pavia, H. Underwater companion planting: competitive interactions between co-cultured multiple seaweed species in land-based aquaculture. *Manuscript*

The following research was conducted during the PhD study duration, but is not included in this thesis:

Thomson, A., Visch, W., Jonsson, P. R, Nylund, GM., **Hargrave, M. S.**, Pavia, H., Stanley, M. Drivers of local adaptation and connectivity along an environmental transition zone in the sugar kelp, *Saccharina latissima. Manuscript*

Glykou, A., Ritchie, K., **Hargrave, M. S.**, Visch, W., Lidén, K. Strontium isotope analysis in prehistoric cod otoliths by laser ablation multi-collector inductively coupled plasma mass spectrometry. *Journal of Archaeological Science: Reports,* 17

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ABBREVIATIONS

AFDW	Ash free dry weight
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
DW	Dry weight
Ek	PSII saturation irradiance
FAO	Food and Agricultural Organization of the United Nations
IMTA	Integrated multi-trophic aquaculture
L. digitata	*Laminaria digitata* (oar weed)
M. edulis	*Mytilus edulis* (blue mussel)
M. gigas	*Magallana gigas* (Pacific oyster)
PSII	Photosystem II
PSU	Practical salinity unit
rETRmax	Maximum relative electron transfer rate
S. latissima	*Saccharina latissima* (sugar kelp)
U. fenestrata	*Ulva fenestrata* (sea lettuce)
WW	Wet weight
WW:DW	Wet weight / dry weight ratio

BACKGROUND & AIMS

The aim of this thesis is to explore the potential for integrated multi-trophic aquaculture (IMTA) systems that do not centre around a fish species requiring external feed and inputs, but rather a combination of non-fed, or extractive, species. More specifically, this thesis focuses on the sugar kelp, *Saccharina latissima,* and to a lesser extent the kelp *Laminaria digitata* and the green seaweed *Ulva fenestrata*, and how the yields and quality of these macroalgae can be improved in co-cultivation with filter feeding bivalves, *Mytilus edulis* and *Magallana gigas*. Further, this thesis also addresses the additive effects of bivalve effluents in IMTA systems where nitrogen is replete, the impacts in IMTA level nitrogen enrichment on mitigating the negative responses of kelp to stress, and finally investigates how nitrogen availability influences the potential for multi-species, or polyculture, seaweed cultivations.

Individual aims of papers:

PAPER I: Mesocosm extractive co-culture: The aim of this initial experiment was to determine the influence of bivalve effluents on the growth, elemental composition and biofouling of *S. latissima*. Nutrient production and filtration of the bivalves, *M. edulis* and *M. gigas* was also quantified, allowing for comparison between the two species.

PAPER II: Field extractive co-culture: This experiment aimed to address whether the strong co-culture dynamics observed in mesocosm studies carry over into open water field cultivations. In this case *S. latissima* droppers were deployed downstream of commercial mussel farms, with particular interest in kelp growth, bioactive compound content and biofouling.

PAPER III: Kelps grown in nutrient repletion in co-culture: A mesocosm system was used to quantify the additive effects of *M. edulis* effluents in combination with artificially enriched nitrogen. The aim of this study was to investigate whether nutrients originating from bivalves influence kelp growth and tissue composition in an environment where nitrogen was non-limiting.

PAPER IV: Nutrient availability and kelp response to stress: The aim of this study was to address how nutrient elevation, for example of the level that may be detected in an IMTA system, may influence the ability of *S. latissima* to respond to and recover from both short and long term hyposalinity stress.

PAPER V: <u>Seaweeds in polyculture:</u> The objective of this study was to explore the potential for multi-species seaweed cultivation, with interest in either synergistic or competitive interactions between species and how nutrient availability influences these interactions.

INTRODUCTION

SEAWEED AQUACULTURE

The global production of cultivated seaweeds is currently estimated at around 30-32 million tonnes (FAO 2017; Naylor et al. 2021) and currently accounts for around 51.3% of total marine aquaculture production in terms of biomass (Chopin and Tacon 2021), with a wide array of uses including as a food source, biofuel, bioplastics and pharmaceuticals (Bixler and Porse 2011; Porse and Rudolph 2017; Shannon and Abu-Ghannam 2019). While seaweed cultivation has been growing rapidly in recent years, by around 8% in the last decade, the use of seaweed as a human food source can be traced back over 14,000 years (Dillehay et al. 2008) and has had an important role in human history. However, currently of the 32 million tonnes of seaweeds produced annually, only around 31-38% is used directly as a human food (Naylor et al. 2021).

Seaweed cultivation is regarded as the least environmentally damaging form of aquaculture (Roberts and Upham 2012), and generally has positive or neutral effects on a range of services (Hasselström et al. 2018). Moreover, seaweed aquaculture provides benefits and ecosystem services that provide a net benefit beyond biomass production. One of the key ecosystem services provided by seaweeds is bioremediation (Neori et al. 2004; Chopin et al. 2012), the uptake of nutrients from the surrounding waters, which, if allowed to accumulate, can result in severe damage to marine ecosystems (Fossberg et al. 2018). For example, cultivation of seaweeds has been employed to successfully counteract eutrophication (Cuomo et al. 1993). There is also evidence for the potential role of farmed seaweeds in carbon sequestration and potential mitigators of ocean acidification (Duarte et al. 2017; Fernández et al. 2019), thus playing a role in climate change mitigation. As such, the cultivation of seaweed holds promise for a wide range of applications, as well as providing an overall positive effect on the marine ecosystem beyond harvestable resources, and thus as a sustainable species for cultivation.

AQUACULTURE AND THE NEED FOR MULTI-TROPHIC SYSTEMS

While capture fisheries have stagnated (Garcia and Newton 1995) and the fishing down of marine food webs has been seen to be unsustainable (Pauly et al. 1998), aquaculture has been filling the deficit in providing food to a growing world population. In fact, aquaculture has been the fastest growing food-production sector globally in recent years (Abreu et al. 2011). In 2014, aquaculture production of fish overtook that of wild fisheries (FAO 2017), and this trend of aquaculture development promises to continue (Fig. 1). In order to develop aquaculture at this rapid rate, great care needs to be taken to ensure sustainable farming practices (Frankic and Hershner 2003). However, many conventional aquaculture practices have caused concern regarding factors such as increasing amounts of nutrient wastes being released in to aquatic

environments (Black 2008; Redmond et al. 2010), where 50-95% of the nitrogen and 80-88% of the carbon input into a fish farm has previously been seen to be lost (Wu 1995). Further, effluent releases rich in nitrogen and phosphorus have been associated with local eutrophication and algal blooms, resulting in dramatic ecosystem damage (Sorokin et al. 1996; Beman et al. 2005; Sanderson et al. 2012). As such, issues surrounding nutrient loading originating from fish farms have seen wide attention.
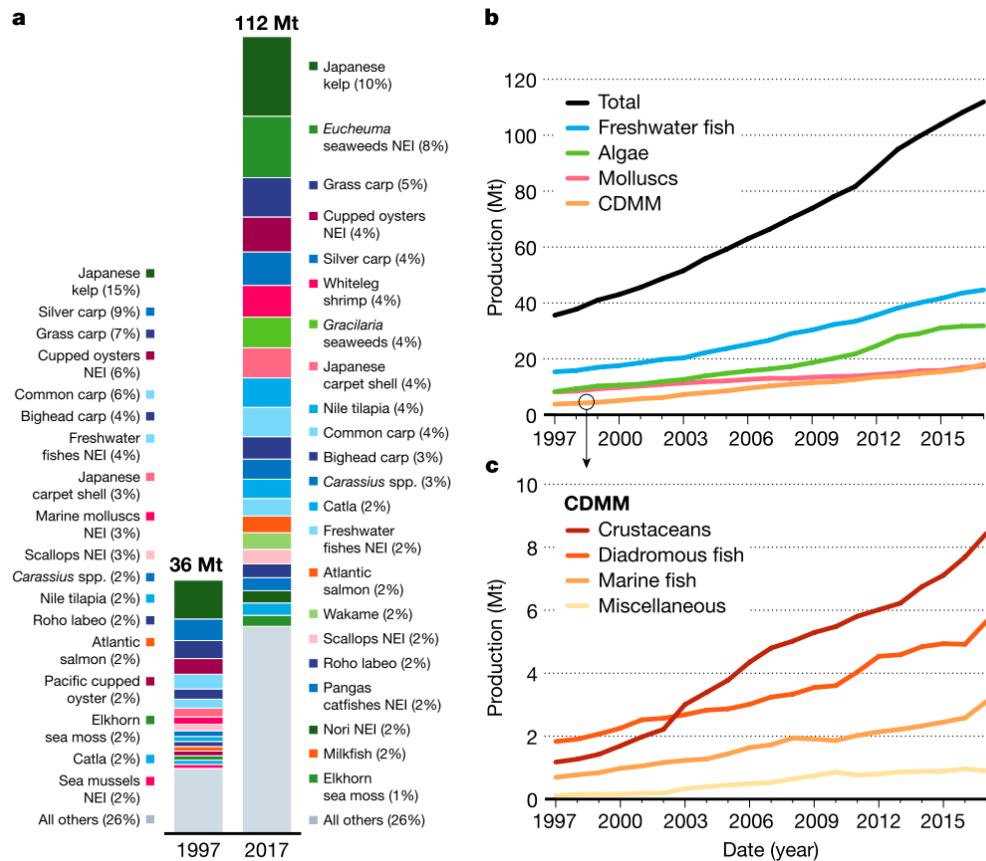

Fig. 1. The growth of aquaculture production from 1997 – 2017, from Naylor *et al.* (2021).

Efforts to mitigate these environmental problems from aquaculture due to nutrient release have involved the incorporation of so called 'extractive' species (Chopin et al. 2001) alongside fish farms (Neori et al. 2004; Alexander et al. 2015). Organisms such as filter feeding bivalves, for example mussels, are used to capture particulate organic matter originating from fish farms (De Silva and Soto 2009), while macroalgae, particularly kelp (Ahn et al. 1998), are incorporated as scavengers of dissolved inorganic waste products (Ridler et al. 2007). These extractive species have a great effect impact as biofilters, where it has been reported that the harvest of farmed seaweeds and shellfish removes around 150,000 tons of nitrogen annually,

as of 2003 (Troell et al. 2003). This incorporation of multiple species from different trophic levels into one farm is known as integrated multi-trophic aquaculture (IMTA). After the development of the concept of IMTA in the USA (reviewed by Park et al. 2018), from the ancient IMTA-like practices in Asia, such systems have been employed globally (e.g. Chopin et al. 1999; Neori et al. 2004; Ridler et al. 2007). IMTA systems vary greatly around the world, since farm design and species use depends greatly on location, season, local species and social factors (Edwards 1998). Despite the variation in format, the concept remains the same: IMTA systems rely on extractive species to cleanse the water of waste products while providing a more diversified farm via additional yields of the extractive species (Chopin et al. 1999; Neori et al. 2004; Troell et al. 2009). As such, it has long been suggested that employing algae and filter-feeding organisms as nutrient scrubbers alongside fed species is the most likely way to improve aquaculture sustainability (Ryther et al. 1975; Cuomo et al. 1997; Troell et al. 1997), particularly in open-water systems (Troell et al. 2003), where it may be the only feasible approach. However, the nutrients that the extractive seaweed species are employed to absorb are not merely waste products, but are essential nutrients that can cause declines in seaweed growth and performance when limiting. As such, these nutrient-rich effluents should be treated as a valuable side stream rather than a potentially harmful waste product. This 'duality of nutrients' must be understood to ensure optimal cultivations and healthy ecosystems (Chopin et al. 2012).

To date, the vast majority of IMTA systems have been strongly focused on, and centred around, fed species. In most cases the focal species is a commercially valuable high trophic level finfish (as reviewed by Barrington et al. 2009). However, extractive species currently make up a slight majority of total global aquaculture production, 50.6% extractive vs. 49.4% fed, (Chopin and Tacon 2021). As such, the potential for co-culture of these widely farmed extractive species, without the involvement of fed species, is potentially interesting.

THE POTENTIAL FOR IMTA SYSTEMS WITH ONLY EXTRACTIVE SPECIES

Unlike finfish culture, filter feeding species such as the blue mussel, *Mytilus edulis*, do not require additional feed or dietary supplements, but instead feed on particulate organic matter naturally occurring in the water column (Grant et al. 2008). As such, they are referred to as 'extractive species' in an IMTA setting, in that they are employed as scavengers of particulate waste (Chopin et al. 2001). Aside from multi-trophic systems, mussels are also cultivated in monoculture, where they provide the same valuable ecosystem service as wild bivalve populations in consuming phytoplankton and removing potentially damaging (if in excess) nutrients such as organic nitrogen from the water column. As such, filter feeding bivalves are exciting as a sustainable farmed species, since, like seaweeds, they require no external feeds and remove nutrients from the water column (Naylor et al. 2021), and thereby also perform a valuable ecosystem service (Gentry et al. 2020).

Despite the fact that bivalves are extractive components of IMTA systems, and provide a net uptake of nitrogen via the consumption of phytoplankton, they also produce their own metabolic waste products. The dissolved fraction of this waste, primarily ammonium (NH_4^+) and phosphorus (PO_4^{3-}) (Dame et al. 1989), released by filter feeding bivalves can accumulate to cause local nutrient elevation and stimulate primary production (Dame and Dankers 1988). From the perspective of seaweed cultivation, nitrogen and phosphorus are the two main limiting factors for growth (Wheeler and North 1981) and nutrient availability can greatly change the yield and composition of a seaweed crop (Harrison and Hurd 2001; Boderskov et al. 2016). The local elevation of these nutrients by bivalve cultivations suggests that there is potential for an IMTA system containing only extractive species, for example bivalves and seaweeds, without the inclusion of a fed species, where seaweeds could benefit from bivalve nutrient enrichment. Such a system would require virtually no external inputs, very little land use, no freshwater requirements and would have an overall positive effect on the local ecosystem.

The potential for such systems was explored in small scale mesocosm experiments in **PAPER I** and at a larger, field scale in **PAPER II**.

IMTA IN NUTRIENT REPLETE CONDITIONS

The general expectation regarding seaweed growth in IMTA systems is that seaweeds benefit from a nutrient fertilisation effect provided by the species they are co-cultured with, since nitrogen is a major limiting factor for macroalgal growth (Wheeler and North 1981). However, the extent to which this fertilisation effect aids seaweed growth when ambient nutrient levels are already high, or the extent to which mussels contribute to nutrient loading in an IMTA system including fish, is unclear. **PAPER III** explores the additive effects of *M. edulis* effluents and artificially elevated nutrients simulating nutrient levels released by intensive fish cultivations. In addition to growth and biomass accumulation, this paper focuses on photosynthetic parameters and pigments to gain insight into the ecophysiological responses of kelp to nutrient enrichment beyond growth and elemental composition.

THE POTENTIAL FOR IMTA TO INCREASE SEAWEED RESILIENCE TO STRESS

Salinity alterations have been identified as one of the three major outcomes of climate change (IPCC, 2007). Thus, salinity stress events are likely to increase in both frequency and severity. It is well known that low salinity (hyposalinity) conditions cause physiological stress in kelp species. This stress leads to reduced growth and therefore a worse crop yield and reduced bioremediation capacity (Nielsen et al. 2016). However, it has been reported that high nitrogen availability in seawater allows kelps to respond better to thermal stress (Fernández et al. 2020). Nitrogen is absorbed and assimilated into amino acids and eventually protein in

seaweed, which is used in growth and maintenance (Harrison and Hurd 2001). The aim of **PAPER IV** is to investigate how nutrient levels produced by IMTA systems may help in increasing kelp resilience to environmental stress, in this case salinity. In this study we investigate the influence of nitrogen enrichment on the mitigation of the negative impacts of hyposalinity in *S. latissima* across both short-term shock and recovery and a longer term acclimation period.

CO-CULTURE OF MULTIPLE SEAWEED SPECIES

Seaweeds are a varied group of organisms with varied applications. Different species yield different useful products, from bioactive compounds used in pharmaceuticals to proteins and carbohydrates required in the human diet. As such, it is an attractive idea to simultaneously grow multiple species of seaweed together in order to yield a diversified crop with a range of uses. However, competitive interactions among seaweed are strong, with competition for light and nutrients, as well as the production and release of allelochemicals to interfere with other species. As such, it is important to understand how different species interact with one-another in polyculture, and how factors such as nutrient level can facilitate or prevent successful multi-species seaweed cultivations. **PAPER V** explores the potential for co-cultivating three commercially important seaweed species and aims to address to what extent combined cultivation of these species may be possible.

METHODS

The experimental work in **PAPER I** took place in a semi-enclosed mesocosm system, pumping water from 1.5m below the sea surface through aquaria units and then back out into the sea. The purpose of this first paper was to verify the concept of a co-cultivation of filter feeding bivalves and kelp on a pilot scale prior to scaling up into open water. Two bivalve species were selected as candidates for the system, the blue mussel (*Mytilus edulis*) and the Pacific oyster (*Magallana gigas*) (Fig. 2), and were secured in mesh bags of 32 individuals, with six bags containing mussels and six containing oysters. These bags were suspended in cylindrical pipes and were supplied with surface seawater at a flowrate of 90-100 L/h. A further six pipes without bivalves were also supplied with surface seawater as control treatments. Water from bivalve and control pipes was then supplied to aquaria containing 10 individuals of the sugar kelp, *Saccharina latissima*, and eventually out of the system (Fig. 3).

Fig. 2. *Saccharina latissima* individual, (~45cm long) on a lightboard ready to be photographed, blue mussels, *Mytilus edulis*, and Pacific oysters, *Magallana gigas*, (each around ~5cm long).

Across the duration of this 12 week experiment water samples were taken downstream of bivalve tanks and kelp tanks in order to quantify the amount of nutrients produced by bivalves

and the amount that was subsequently absorbed by the kelp. At the end of the experiment kelp were measured to provide elongation, via holepunch tracking (Parke 1948), and biomass growth rates, photographed for digital quantification of epiphyte coverage and finally freeze dried, homogenized and analysed for elemental tissue composition and stable isotope ratios of carbon and nitrogen.

The experiment in **PAPER II** was conducted in open-water field conditions near Mollösund, Orust, on the Swedish west coast. This study involved the deployment of juvenile *S. latissima* sporophytes (5-10mm in length) downstream of commercial mussel farms run by Scanfjord AB, Sweden's largest blue mussel producer. The age of the mussel farms at the commencement of the experiment was 12-18 months, meaning that the mussels ropes had high levels of coverage with large mussels, but would not be harvested during the experimental duration, since they are of harvestable size after around two years.
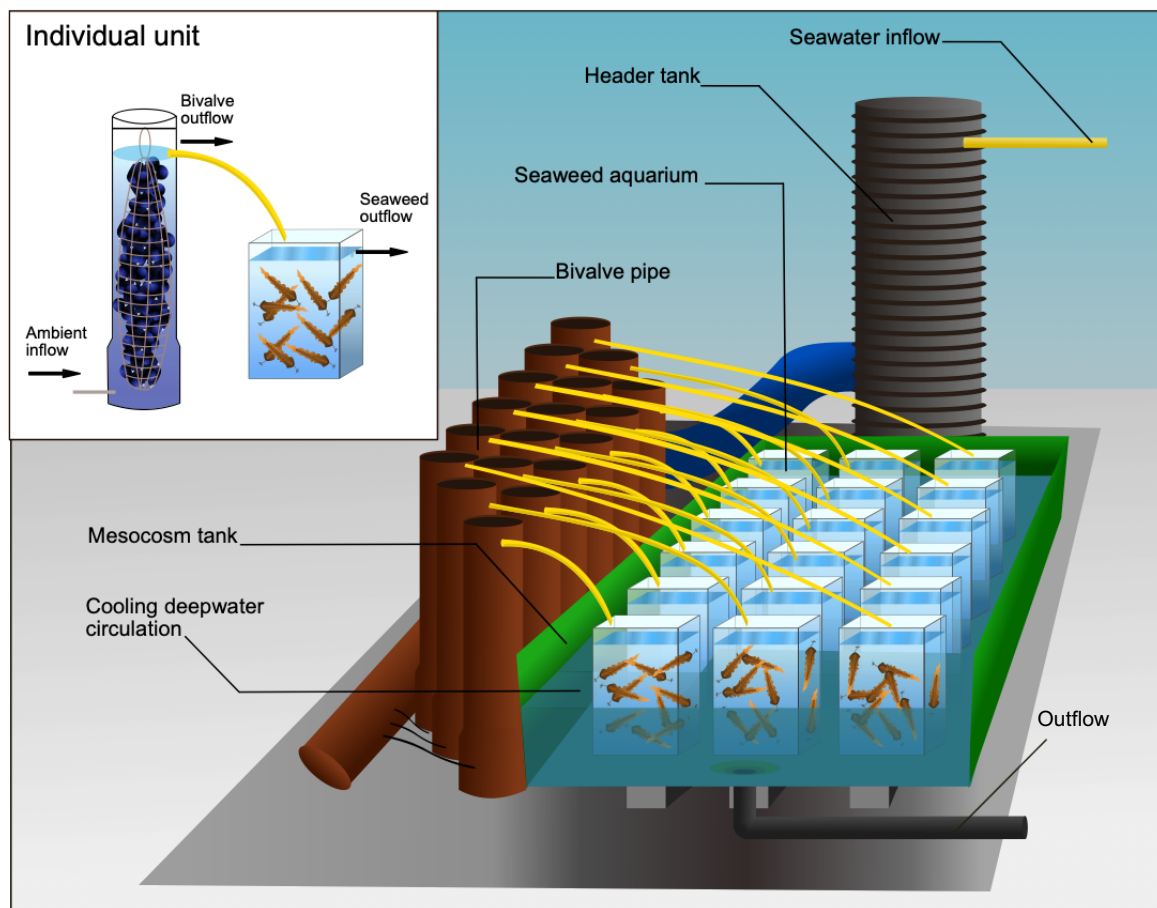

Fig. 3. Diagram of the mesocosm system used in **PAPER I** showing the entire system containing all replicates of bivalve pipes and seaweed aquaria, as well an individual experimental unit outlining how seawater was pumped though bivalve aggregations and subsequently to kelp tanks before flowing out of the system. 'Bivalve outflow' and 'Seaweed outflow' indicate where water samples were taken on a daily basis.

Adult individuals of *S. latissima* were collected from within 1km of the mussel farms prior to the experiment and were returned to Tjärnö Marine Laboratory where they were induced to produce fertile tissue. Spores were released from these parent plants, grown vegetatively as gametophytes in the lab, sprayed on coils of nylon string and out planted near Mollösund, either 10m downstream of mussel farms (Fig. 4), or at control sites at least 500m away from the nearest mussel farm unit. Kelp lines of 1m length were cut from coils and attached to vertical droppers at either 1-2m or 3-4m deep. Each farm or control site received a total of 12 kelp droppers, with six containing kelps attached at 1-2m and a further six at 3-4m (Fig. 5). After deployment at the start of November, kelp lines were left in the water until the start of May when they were harvested. Light and temperature loggers were deployed at each site at depths of 3-4m.

Fig. 4. Kelp droppers (white buoys) deployed downstream of mussel farms (large grey buoys and yellow hazard markers) off Mollösund, Orust, in November. The whole mussel farm unit (roughly 200 x 20m) is not pictured. In this image the wind is blowing the buoys against the mean direction of the water current.

In May, 50 lines , out of the 72 lines that were deployed, were recovered after a stormy winter. However, the loss of some lines was anticipated and mitigated for by the deployment of many units. All gear was removed from the water and the 1m long segments of line to which kelp were attached were removed and transported back to Tjärnö Marine Laboratory. Kelps were weighed from each line (detached in their entirety) to provide a measurement of wet biomass in terms of kg/m. From each line 10 kelp individuals were randomly selected and their length

measured. A further three kelps from each line were randomly selected and photographed, for later quantification of epiphyte coverage, and were then immediately frozen prior to freeze drying for pigment and elemental composition analysis.

After freeze drying, kelp samples were homogenized in preparation for pigment analysis (Fig. 6). 50mg of kelp tissue was extracted in 5ml Acetone (90%) over 60 minutes, with 45 minutes of vigorous shaking and 15 minutes of ultrasonication. Samples were then spiked with an internal standard (apo-carotenol) before injection into the HPLC column. Standard curves were calculated prior to quantification of kelp pigment content for all pigments.
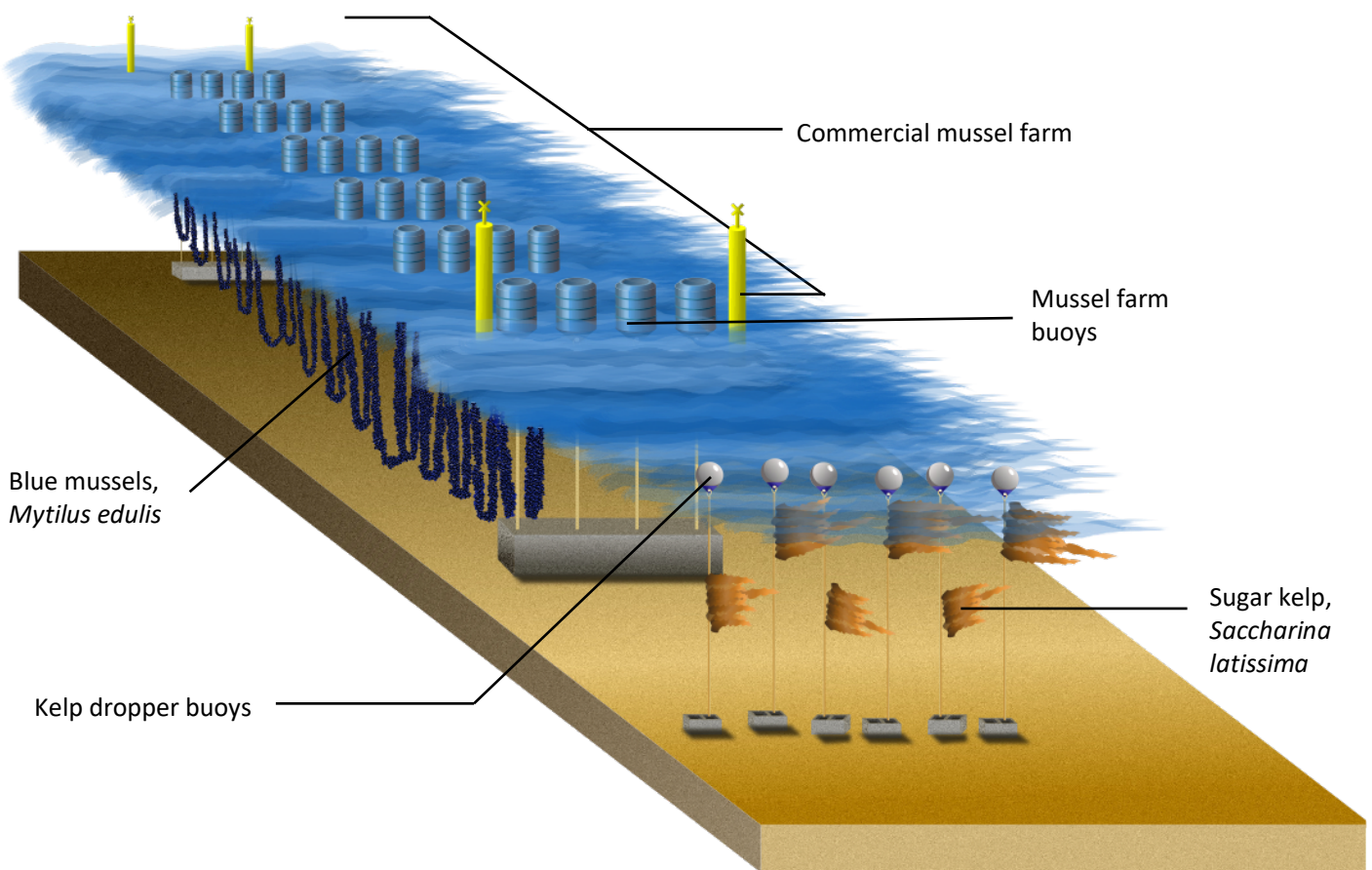
Fig. 5. Diagram of the IMTA system used in **PAPER II** indicating vertical droppers with *S. latissima* seeded lines, as well as the design of the mussel farms, where mussels settle and grow attached to longlines suspended from large buoys.
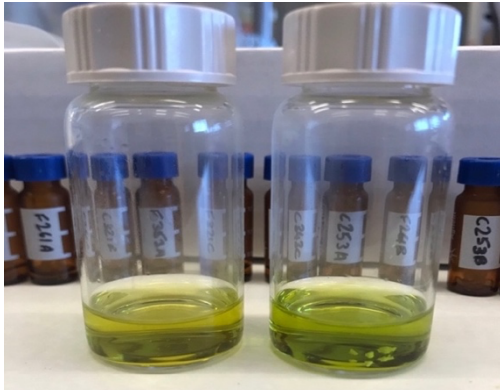
Fig. 6. *S. latissima* pigment extracts ready for HPLC analysis. Differences in pigment content are often visible to the naked eye prior to analysis.

PAPER III was a return to the mesocosm system of **PAPER I** with alterations to design (Fig. 7). One year old *S. latissima* individuals were collected from a nearby commercial scale seaweed farm as study plants for the experiment. These kelp were subjected to one of three experimental treatments: ambient surface seawater (control), surface seawater enriched with ammonium nitrate (NH_4NO_3) to around 3-4µM, and enriched surface seawater and effluent from aggregations of 50 blue mussels.

The ecophysiological responses of interest in this study were kelp growth (elongation and biomass), photosynthetic activity (electron transfer rate and saturating irradiance, rETRmax and Ek), elemental composition (carbon, nitrogen and carbohydrate content) and pigment content (chlorophyll *a,* total carotenoids and fucoxanthin).
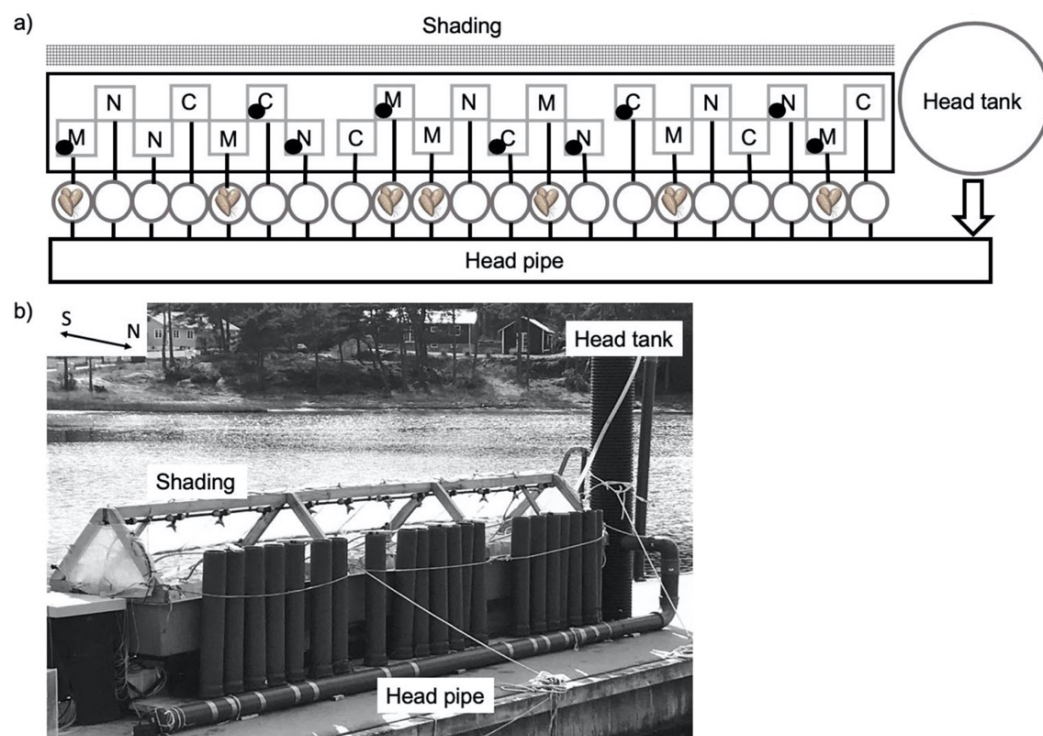

Fig. 7. Schematic of the experimental setup and photograph of the mesocosm system. Images: Luca Rugiu.

PAPER IV was conducted under laboratory conditions in a temperature controlled room. Juvenile sporophytes were selected from tank cultivations of second generation *S. latissima* whose parents were collected nearby in the Kosterfjord. This study involved two separate experiments, one to investigate short term shock and subsequent recovery from hyposalinity stress, and the other to study acclimation over a longer period (10 days).

To assess how prior nitrogen availability influenced the ability of *S. latissima* to recover from salinity stress, kelps were treated with either ambient or enriched (15µM NH$_4$NO$_3$ pulse every 24h) seawater. After five days of pre-treatment kelps were subjected to a 24h exposure to hyposaline water with a salinity of 10PSU prior to a return to ambient salinity (28PSU). The quantum yield of the kelp was analysed immediately after return to ambient salinity (0h) and then at 2 hour intervals until 28h of recovery.

Fig. 8. Five *S. latissima* individuals in laboratory tumble culture. Coloured ties attached to the stipe allow individuals to be identified for repeated analysis.

The acclimation study involved a crossed design of two experimental factors, resulting in four experimental treatments of either ambient nitrogen and elevated nitrogen (ambient nitrogen + ambient salinity (AN+AS), high nitrogen + ambient salinity (HN+AS), ambient nitrogen + low salinity (AN+LS), and high nitrogen + low salinity (HN+LS). Low salinity levels were half of ambient seawater salinity on the day of tank refreshment (where ambient salinity ranged from 27.2-30.6 PSU), which occurred every 24h, and high nitrogen levels were a result of addition of 15µM NH$_4$NO$_3$ at every refreshment. Measurement parameters in this acclimation study were growth (elongation, biomass and width), photosynthetic rate (electron transfer rate (ETR), saturating irradiance (Ek) and pre-saturation slope angle (alpha)), photosynthetic pigments (chlorophyll *a,* fucoxanthin, xanthophylls (violaxanthin and zeaxanthin) and ß-carotene) and elemental composition (carbon and nitrogen).

PAPER V was also conducted in an outdoor mesocosm system refreshed with surface seawater (Fig. 9). In this experiment we aimed to assess the potential for growing multiple seaweed species together, in a concept known as polyculture or companion planting. The kelps *S. latissima* and *Laminaria digitata* were collected from a sea-based seaweed farm, while the green alga *Ulva fenestrata* was obtained from tank cultivations. As such, three algal species were used and were either grown in polyculture (all three together) or individually as monocultures. Additionally, all of these culture conditions were then subjected to two

nutrient treatments, either receiving ambient nitrogen in surface seawater or artificially elevated nitrogen (pulses ~20 µM/L ammonium, NH₄Cl) every 48 hours. The experiment lasted four weeks, during which periodical growth measurements and water samples, for quantification of nutrient uptake, were taken. After the four week duration, kelp were assessed for growth (elongation and biomass), photosynthetic rate (electron transfer rate (ETR) and saturating irradiance (Ek) and elemental composition (carbon and nitrogen).
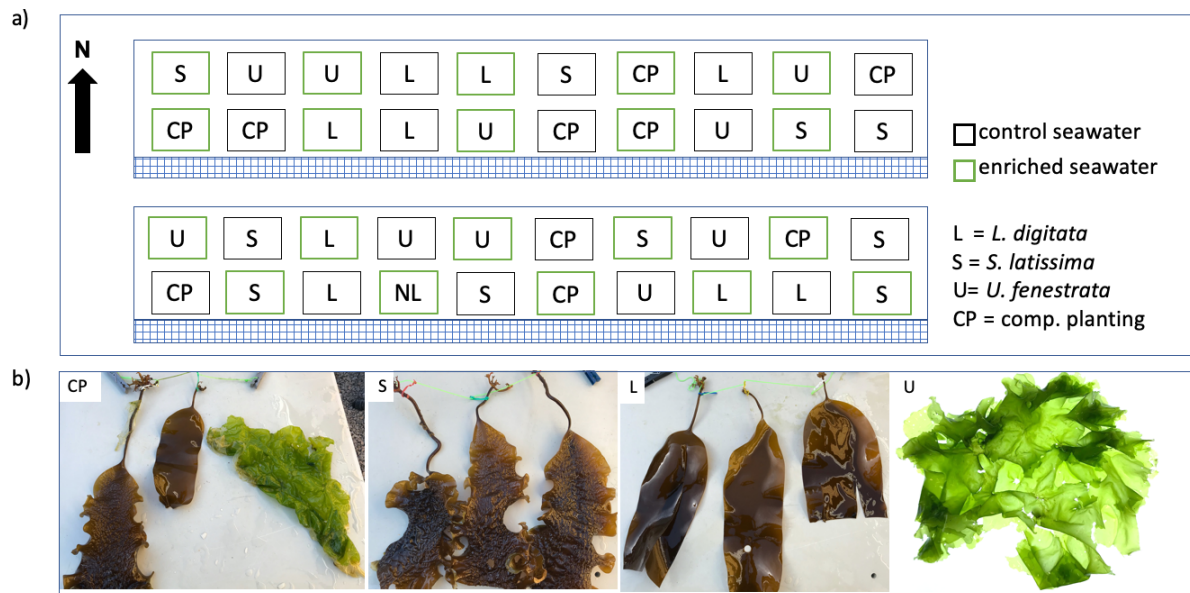

Fig. 9. a) Schematic of the experimental system and design, b) the different algae combinations for companion planting (CP) and monoculture treatments (S, L & U). Images: Luca Rugiu.

KEY RESULTS AND DISCUSSION

Overview of key finding

The main results of the thesis in relation to the previously outlined specific aims were:

PAPER I: The results show a strong potential for the co-cultivation of filter feeding bivalves and kelps in tank culture and suggest the potential for this concept to be scaled up into field conditions. The bivalves not only elevated nutrients which resulted in a fertilisation effect for the seaweeds, but also acted as a biofilter, removing particles from the water resulting in reduced epiphyte coverage on kelps.

PAPER II: This field study provides evidence that the effects of co-culture, that were identified in mesocosm experiments, carry over into the field and can be successfully upscaled. Kelp growth was enhanced in co-culture with blue mussels and kelps again had reduced epiphyte coverage, both of these results appear to have been primarily driven by biofiltration by mussel farms rather than nutrient enrichment.

PAPER III: The key finding in this study was that, although co-cultivation had no significant effect on kelp growth when background nitrogen levels were also high, the bivalve effluents still influenced seaweed photosynthetic parameters and pigment content. Thus, a co-cultivation of kelps and bivalves is beneficial regardless of background nitrogen levels.

PAPER IV: The results of this paper indicate that the negative physiological effects of short term hyposalinity shock and subsequent recovery are ameliorated by prior exposure to IMTA level nitrogen enrichment. Moreover, from the perspective of acclimation, while parameters such as growth are negatively affected by hyposalinity despite nutrient level, nitrogen enrichment aids in other aspects such as photosynthetic rate and internal stores.

PAPER V: At ambient nutrient levels, competition for nitrogen results in sub-optimal seaweed growth in polyculture compared to monoculture. However, these negative interactions are to some extent neutralised by nutrient elevation, although allelochemical interactions still may have an effect.

NON-FED MULTI-TROPHIC AQUACULTURE SYSTEMS

Papers **I** and **II** explored the potential for IMTA systems containing solely non-fed, or extractive, species. The studies involved the combination of filter feeding bivalves and kelp, with the aims of enhancing kelp yields via mussel by-products and alterations to water conditions. Both of these studies highlighted the potential of such systems, with beneficial outcomes such as enhancements in kelp biomass quality and quantity. However, while the outcomes of both experiments were similar, it appears that the driving factor for enhancing kelp productivity differed between the two studies. Overall, the driving factor in small scale mesocosm in **PAPER I** was the elevated nutrient conditions by bivalves, while the main driver in the open water cultivations in **PAPER II** was via bivalve biofiltration.

In **PAPER I** the levels of nitrogen and phosphorus, in the forms of ammonium and phosphate, were significantly elevated by bivalves in comparison to the ambient nutrient levels in control treatments.
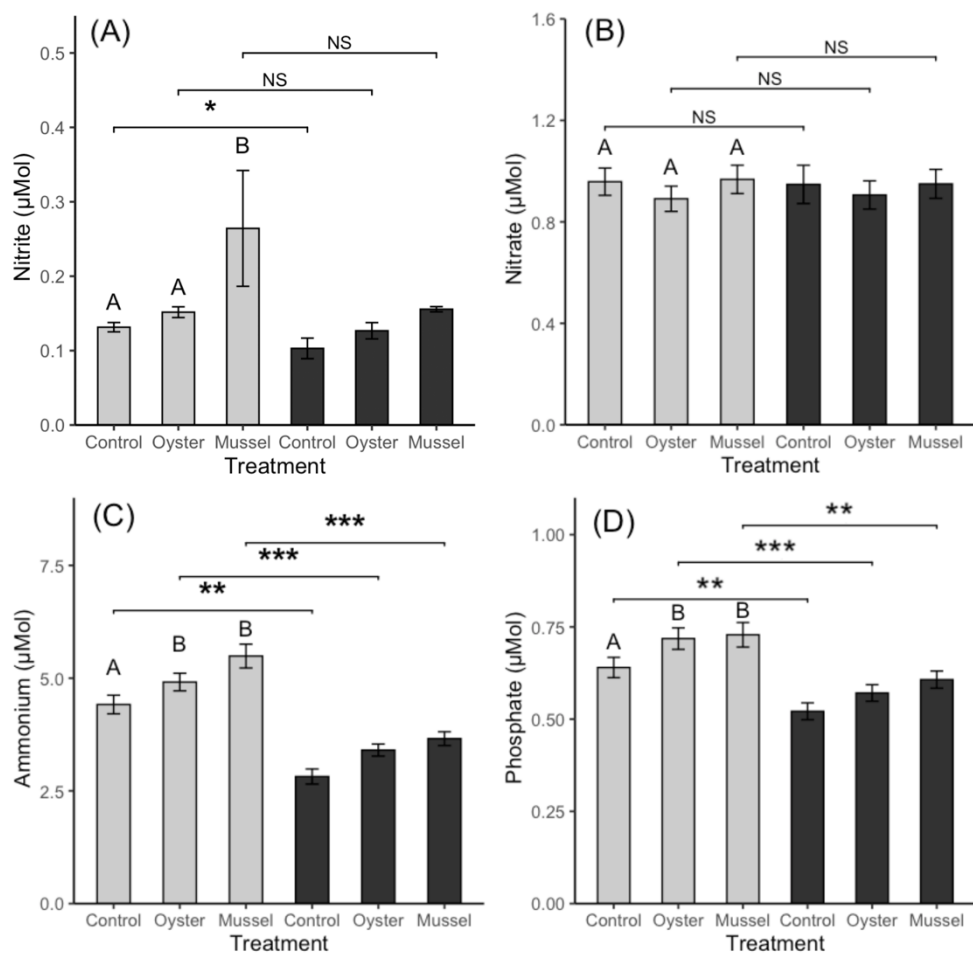

Fig. 10. Mean nutrient elevation by bivalves (light grey bars and subsequent levels after seaweed nutrient uptake (dark grey bars) in **PAPER I.**

While the aggregations of bivalves used in this study were small, 32 individuals per experimental unit (see Fig. 3), the ammonium elevations were around 1μM over the experimental duration on average, but were observed to be boosted by up to 6μM at certain time points.

In the cases of ammonium and phosphate, we also observed a reduction of these nutrients in the outflow water from kelp aquaria, indicating kelp uptake. The use of nitrogen sources originating from bivalves rather than ambient seawater is visible in nitrogen stable isotope ratios, where $\delta^{15}N$ values in kelp from co-cultivation treatments is lower than that of kelps grown in monoculture, indicating use of the bivalve-originating pool of ^{14}N enriched nitrogen. However, despite nutrient enrichment, no variance in *S. latissima* growth rate was observed.
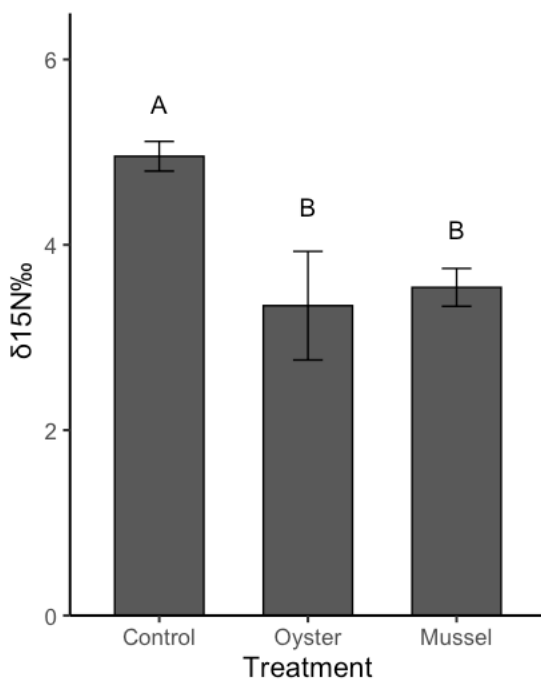

Fig. 11. $\delta^{15}N$ ratios of kelp grown in association with Pacific oysters, blue mussels or in ambient seawater. Significant difference in values between control treatments and bivalve treatments indicates kelp use of different nitrogen pools in monoculture vs. co-culture.

Beyond our initial hypothesis that kelp would benefit from dissolved nutrient production by bivalves, we also observed a decline in the amount of epiphytes on kelp blades grown in co-culture with bivalves. We interpret this result as being a result of bivalve feeding, which involves the filtration of suitable particles out of the water column as a food source. This idea is supported by the observed reduction of chlorophyll *a* levels downstream of bivalve pipes in comparison to inflowing seawater. Chlorophyll *a* concentration in the water is a good proxy for phytoplankton abundance. From these data we can infer that the mussels and oysters in our system are consuming plankton and particles from the water column that may be potential fouling species, thus resulting in cleaner kelps in co-culture rather than monoculture.

The aim of **PAPER II** was to explore to what extent the bivalve – kelp interactions observed in small scale experiments carried over into open water conditions, with different hydrological conditions and dilution rates of nutrients. Although the initial expectation was that the effects observed in mesocosm studies would be reduced in open water cultivations, the results indicate that these effects were in fact strengthened (Fig. 12).
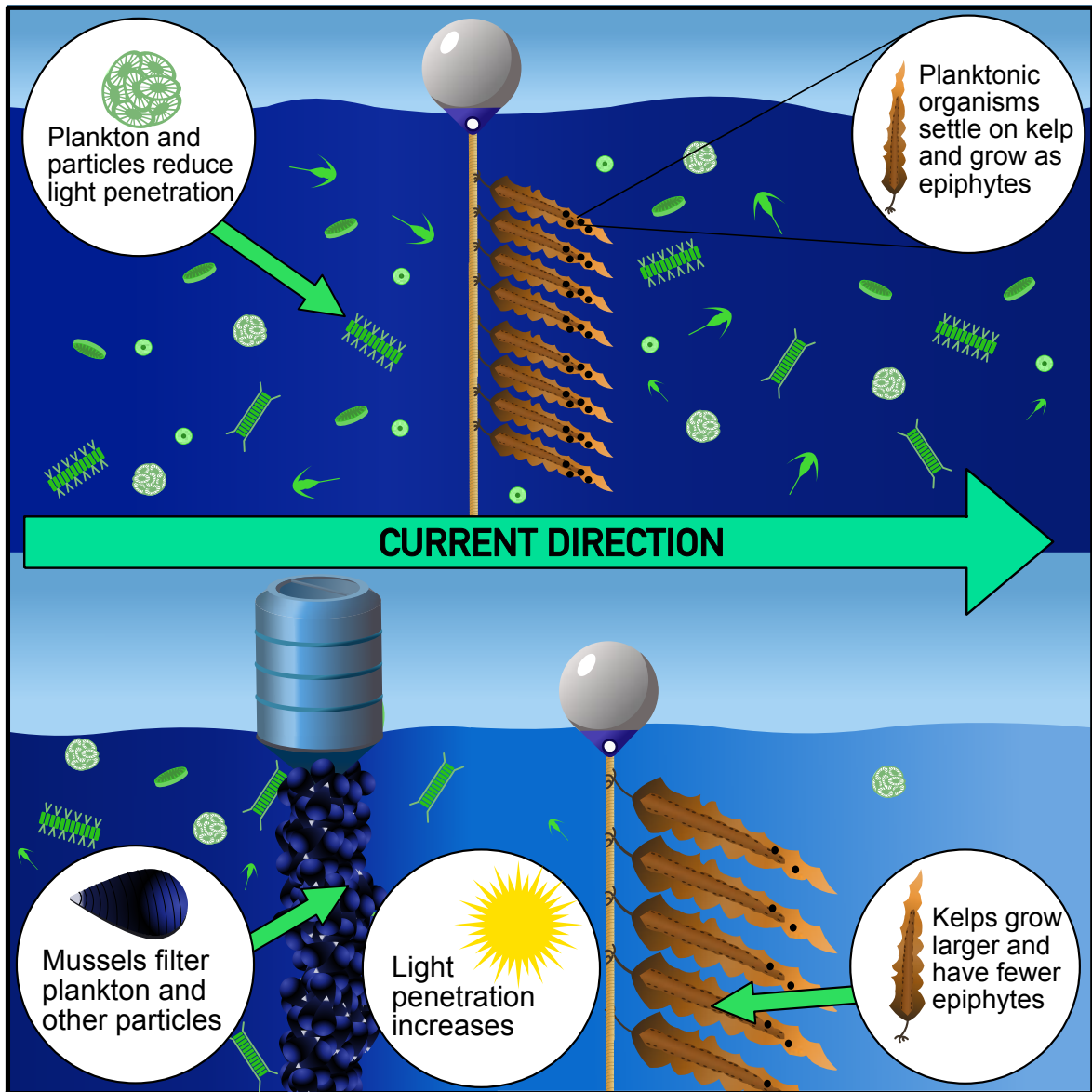

Fig. 12. Graphical abstract summarising the key findings of paper II, in which kelp was grown in monoculture (upper half) or in co-culture with commercial mussel farms (lower half).

Kelps grown downstream of mussel farms grew larger at both depths than kelps grown in monoculture, with comparable growth being achieved at 3-4m deep in co-culture as at 1-2m in monoculture (Fig. 13). This trend was also observed in wet biomass (kg/m^1). Thus, in this field scale study, growth was increased by co-culture despite not being observed at the smaller experimental scale. As was seen in **PAPER I**, epiphyte coverage was drastically reduced on kelp blades in co-culture treatments, from 15% coverage in monoculture at 1-2m to around 6% in co-culture conditions at the same depth.
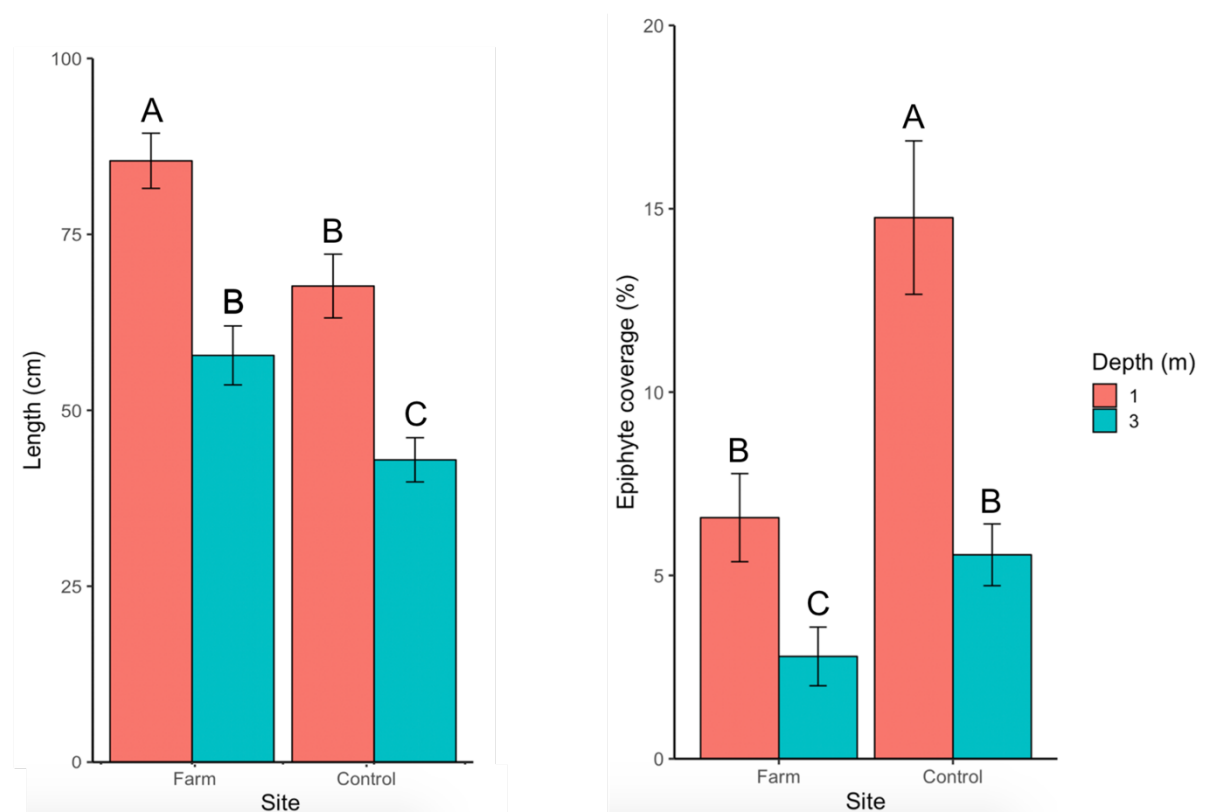

Fig. 13. Mean kelp length and epiphyte coverage after harvest of kelps grown in either co-culture with mussel farms or in monoculture conditions at control sites, at two depth levels.

Despite the initial hypothesis that kelp would benefit from nutrient emissions originating from mussel farms, we observed no significant variation in kelp nitrogen content or stable isotope rations to provide evidence for this. Instead, based on data from light loggers (Fig. 14), it appears that the driving factor for enhanced kelp growth was an increase in light intensity downstream of mussel farms as a result of biofiltration from the large aggregations of mussels in the farms. Such a strong biofiltration effect is also supported by the drastic reductions in epiphyte coverage in kelp grown in co-culture. While there is a wealth of research reporting the scale of nutrient elevation by filter feeding bivalves, both wild and cultivated, it appears that in this case any nutrient enrichment had no detectable influence on seaweed growth. It is possible that the dilution of nutrients in open water systems is very rapid, and these

nutrients originating from farms may have dispersed before being available to kelps grown only 10m downstream. One alternative explanation could be that any internal nitrogen stores in the kelp were utilised in growth during spring and thus internal reserves were depleted. However, if this were the case, we would still expect to see a variation in isotopic signature between monoculture and co-culture kelps, which was absent. Lastly, the coastal area around Orust is regarded as at risk of eutrophication due to high nutrient levels. This may explain the apparent lack of a fertilisation effect upon kelp grown in co-culture in comparison to monoculture kelps, as nitrogen may not have been limiting for growth in either cultivation treatment.
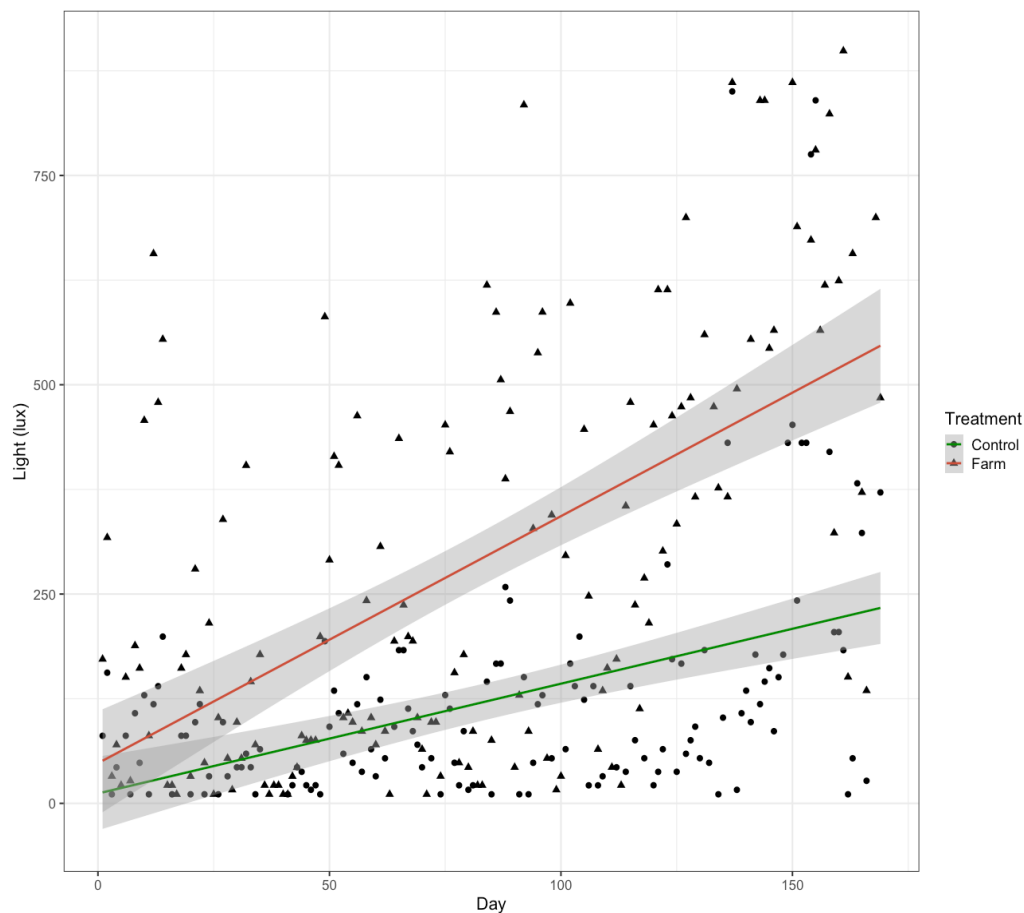

Fig. 14. Linear regression models of light intensity at 3m downstream of mussel farms (orange line, triangular points) and at control sites (green line, circular points) from January 1ˢᵗ and May 1ˢᵗ. Grey shading indicated 95% confidence intervals.

Further evidence for enhanced light intensity driving kelp growth is clear in kelp pigment content, particularly at 3-4m deep in co-culture, where content of chlorophyll *a*, fucoxanthin and phaeophytin were elevated. The higher levels of these pigments at the deeper depths provides evidence for differing light regimes downstream of mussel farms compared to monoculture sites.

CO-CULTURE OF MUSSELS AND KELP IN HIGH NITROGEN CONDITIONS

In papers **I** and **II**, the potential for IMTA systems containing only extractive species was explored. The impacts of filter feeding bivalves on kelp growth was clear at both mesocosm and field scales, with the influence of biofiltration and nutrient production being key factors. However, the absence of a nitrogen enrichment effect in **PAPER II**, potentially explained by sufficiently bioavailable nitrogen in ambient seawater, suggests that the effects of nutrient enrichment may be nullified if nitrogen is not limiting for growth.

PAPER III aimed to address to what extent blue mussel effluents contribute to kelp growth and ecophysiological parameters in an IMTA system where background nitrogen levels are high and non-limiting. As hypothesised, kelp growth was enhanced by artificial nutrient loading, but did not significantly increase further with the simultaneous provision of bivalve effluents (Fig. 15). Similarly, photosynthetic parameters and internal carbohydrate storage was not significantly influenced by the additive effects of mussels.
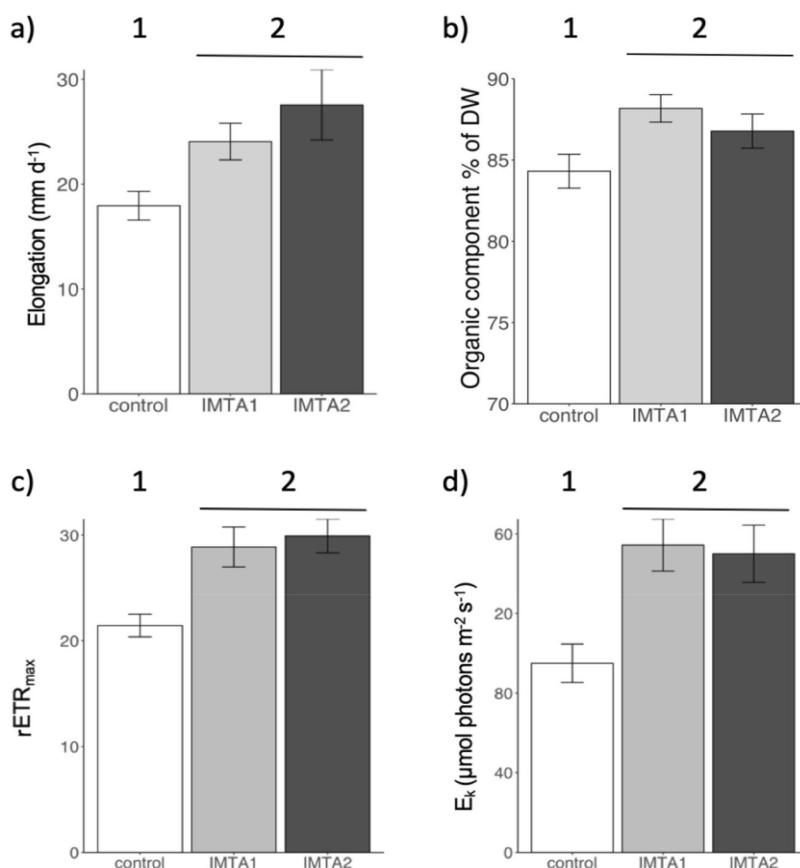

Fig. 15. Growth, organic content and photosynthetic parameters of kelp supplied with ambient seawater (control), artificially elevated nutrients (IMTA 1) and elevated nutrients + mussel effluents (IMTA 2). Numbers above plots represent groupings by statistical significance.

However, although growth and photosynthetic parameters of *S. latissima* were not significantly affected by the additive input of mussel effluent, significant alterations in pigment content were observed. Pigments were seen to increase in comparison to controls via

simultaneous nutrient enrichment and mussel effluent, whereas nutrient enrichment alone did not significantly alter pigment content beyond levels in controls (Fig. 16).

Fig. 16. Pigment content (chlorophyll *a*, total carotenoids and fucoxanthin) in *S. latissima* blades after 5 weeks of exposure to IMTA treatments.

While it has been observed that the pigment content of many seaweed species is driven by nutrient availability, these studies have generally observed such responses under high levels of nutrient loading. In this study we demonstrate that small elevations (3-4µM) drive significant enhancements of pigment content. Such levels are representative of nutrient loading downstream of fish farms. However, we also present evidence for the additive effects of bivalve effluents, which led to a further increase in pigment content due to a further relatively small nutrient enrichment effect (~2µm ammonium and ~0.07µm phosphate).

The additive effects of bivalves and fish farm nutrient elevation in increasing pigment content above solely fish farm enrichment indicates that kelp continue to have a bioremediative effect beyond nutrient conditions that are saturating for growth and photosynthetic rate. Moreover, the additive nutrient enhancement by mussels in IMTA systems is clear, providing more evidence for bivalves as local nutrient emitters as well as a purely 'extractive' component of IMTA systems. However, while the additive effects of mussels did not enhance growth in this study, the investment of surplus nutrients by kelp into photosynthetic pigments resulted in a crop with a higher content of bioactive compounds, and thus a more valuable crop yield.
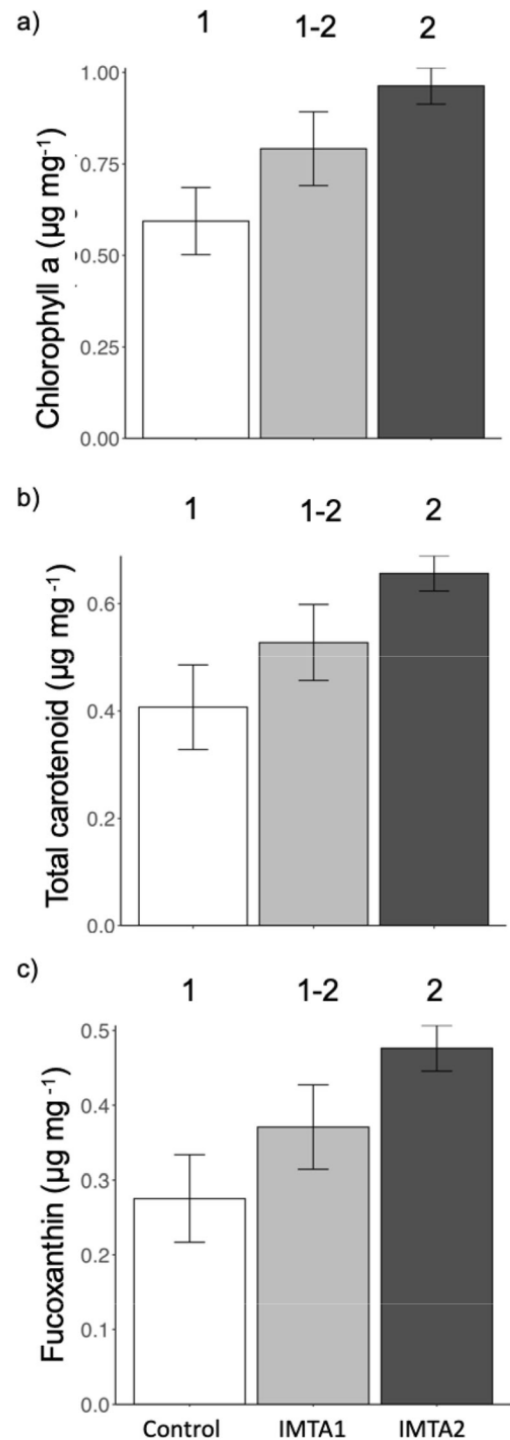
THE POTENTIAL FOR CO-CULTURE IN RESISTING ENVIRONMENTAL STRESS

Marine surface salinity fluctuations can be extreme, especially in coastal marine environments where seasonal freshwater inputs can result in hyposalinity events (Karsten 2012), particularly due to runoff from rivers, and glacier and sea ice melt (Hanelt et al. 2001). More unpredictably, on a global scale climate change is driving shifts in precipitation patterns, which are leading to increased rainfall in some areas (Trenberth 2011). While seaweed species, such as kelps, can generally tolerate a range of salinities, low salinity can cause declines in growth and physiological health (Spurkland and Iken 2011; Møller Nielsen et al. 2016). Kelp species grown in aquaculture are particularly susceptible to these hyposalinity events, since they are generally cultivated close to the sea surface and in coastal, near-shore waters. The Skagerrak is particularly prone to seasonal salinity fluctuations, as can be seen in the long-term dataset at Tjärnö Marine Laboratory (Fig. 17).
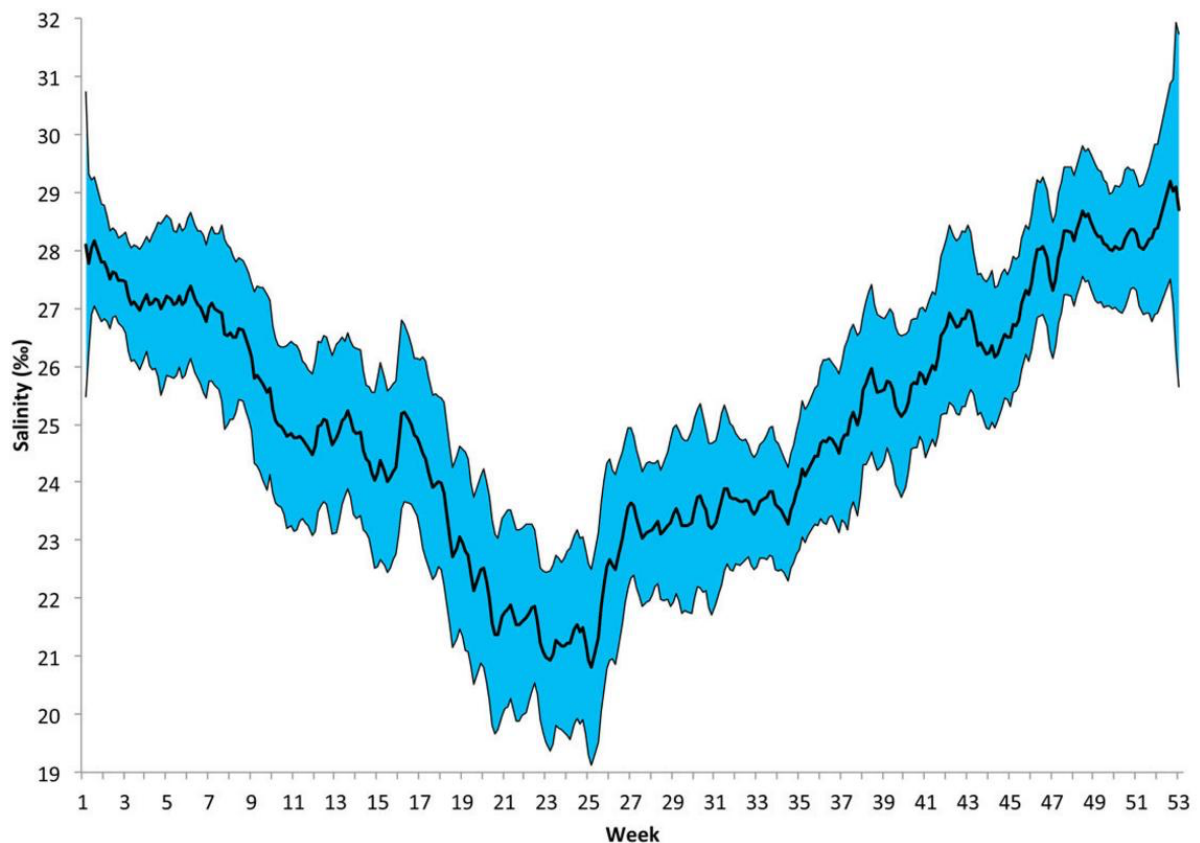

Fig. 17. Mean annual salinity (1980-2013) at 1m depth at Tjärnö Marine Laboratory, mean (bold line) with 95% confidence interval (blue). Highest and lowest measured values are 34.5 ‰ and 9.3 ‰, respectively. Image: Martin Larsvik.

With the aim of investigating how IMTA-level nitrogen enrichment my aid kelp in responding to and tolerating low salinity stress, **PAPER IV** involved experiments of both shock and recovery, and acclimation.

In terms of shock and recovery, after 24h of exposure to hyposalinity (10PSU) and a subsequent return to ambient salinity (28PSU), kelps that had been pre-treated with low level nitrogen elevation recovered faster, as well as declining less severely in terms of quantum yield (Fig. 18). While quantum yield does not inform us exactly what the cellular responses of kelp to hyposalinity are, it provides an insight of overall algal performance, and thus is a useful measurement for this type of study.
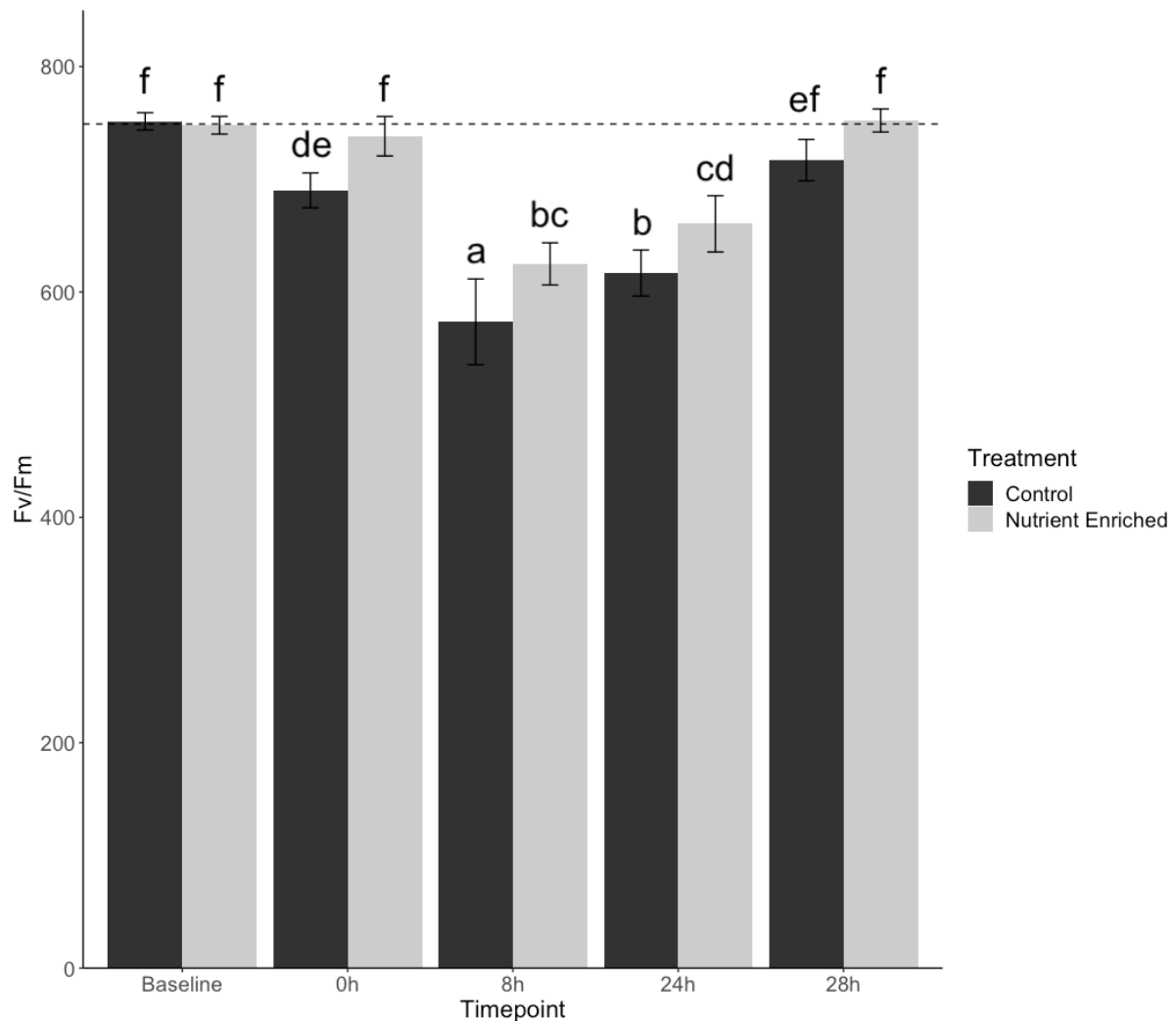

Fig. 18. Quantum yield (Fv/Fm) of *S. latissima* pre-treated with either ambient nitrogen (dark grey bars) or elevated nitrogen (light grey bars) prior to hyposalinity exposure (baseline) and after 24h exposure to hyposalinity conditions and subsequent return to ambient salinity. Letters above bars indicate significant difference.

While the benefit of nitrogen enrichment in enhancing the resilience of *S. latissima* to hyposalinity stress was evident on the short term, nitrogen – salinity stress interactions were more variable with regard to acclimation. Some parameters, such as growth in terms of elongation, appeared to be driven primarily by salinity, whereas photosynthetic parameters appear to be solely driven by nitrogen level. In general, there was no interaction between the two experimental factors of salinity and nitrogen.
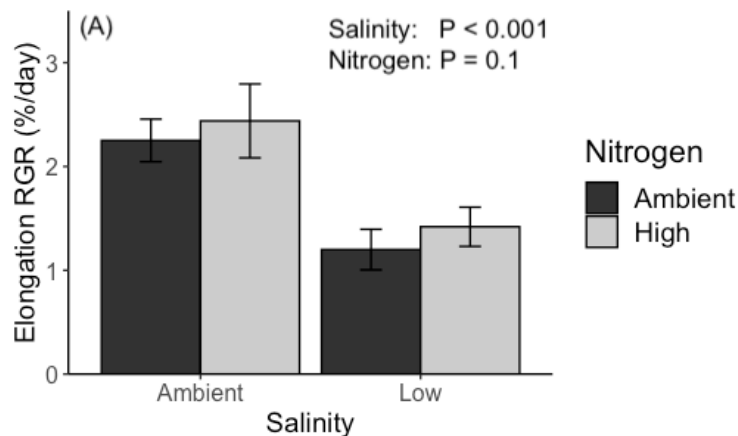

Fig. 19. Growth rates of *S. latissima* over the 10 day experimental period exposed to varying salinity and nitrogen levels, annotations indicate which factors effected growth.

S. latissima growth in both nitrogen treatments declined by around 50% under hyposalinity conditions, with no significant influence of nitrogen level on growth in any treatment (Fig. 19). This data indicates that hyposalinity stress results in drastic declines in kelp growth that is not ameliorated by increases nitrogen level. The absence of significant enhancements in kelp growth under enrichment nitrogen and ambient salinity compared to kelps in ambient nitrogen and salinity treatments is a surprising result. It is possible that nitrogen was not limiting for growth under ambient conditions for these small, juvenile kelp.
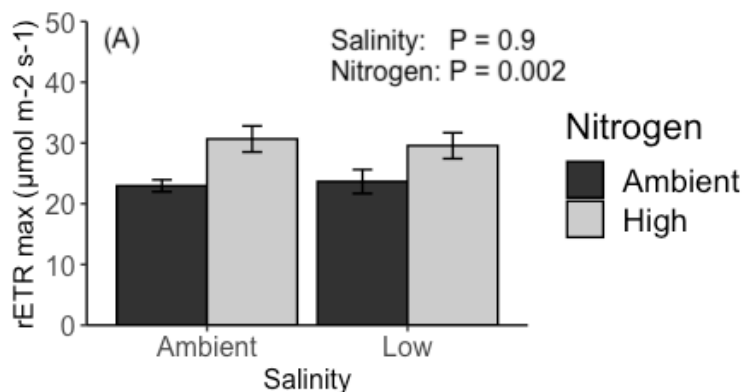

Fig. 20. Maximum electron transfer rate of *S. latissima* after the 10 day experimental period exposed to varying salinity and nitrogen levels, annotations indicate which factors effected ETR.

S. latissima showed significant difference in ETR at the end of the 10 day experimental exposure as a result of nitrogen level, but was unaffected by hyposalinity stress (Fig. 20). This was also the case for Ek, the saturating irradiance for photosynthesis. The absence of a salinity

driven effect of ETR and Ek suggests that hyposalinity stress had no damaging effect on kelp photosynthetic apparatus, while nitrogen elevation enhanced photosynthesis. Such elevation of photosynthetic rate, as a result of increases in nitrogen availability, have been previously reported (Abreu et al., 2009; Kang et al., 2009, Rugiu et al. 2020).
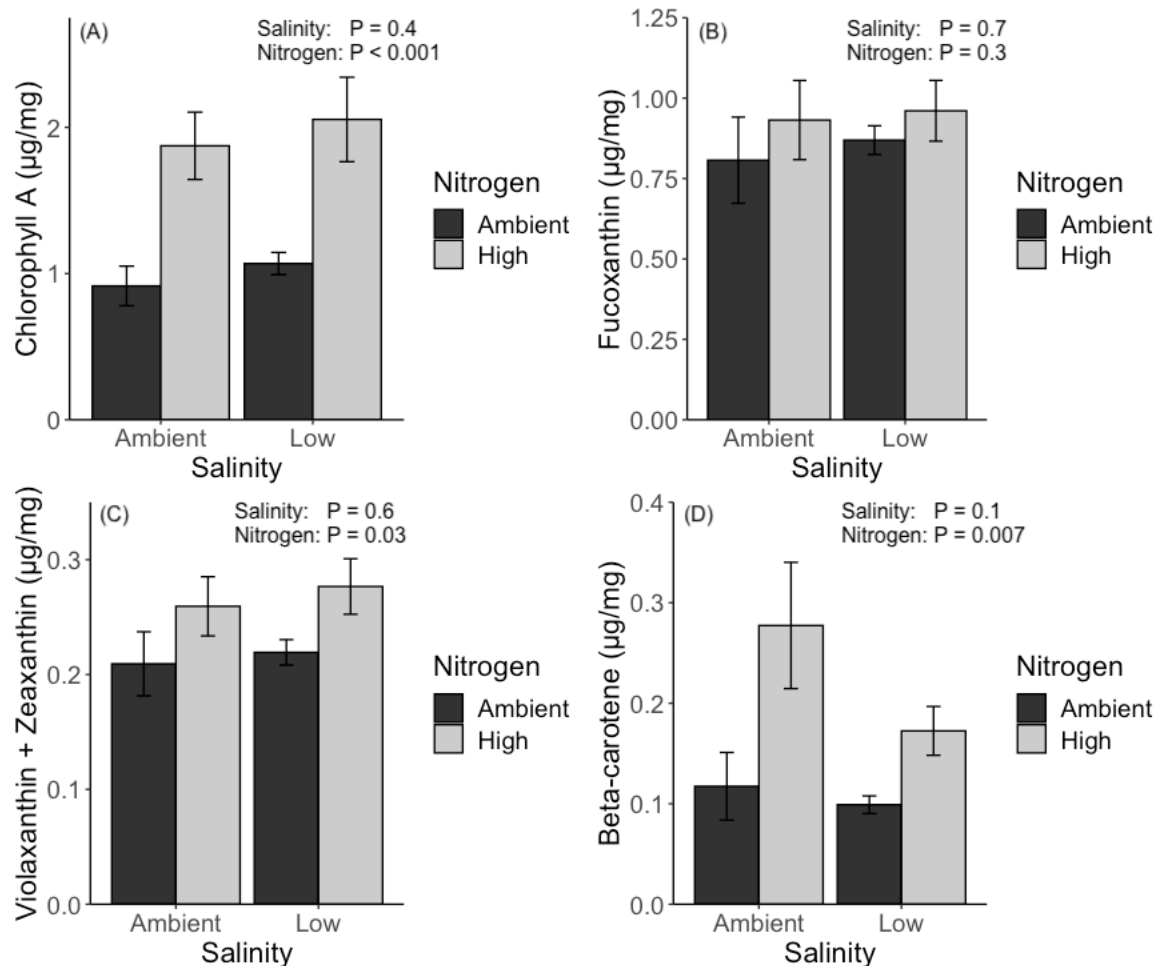

Fig. 21. Photosynthetic pigment content in *S. latissima* at the end of the 10 day experimental period exposed to varying salinity and nitrogen levels. Annotations indicate which factors effected kelp pigment content.

Similarly to the photosynthetic rate parameters, photosynthetic pigment content was driven solely by nitrogen level (Fig. 21). This was the case for chlorophyll *a*, ß-carotene, as well as the sum of the xanthophyll pigments violaxanthin and zeaxanthin, while fucoxanthin was unaffected by either treatment or combination. These findings provide more evidence for the photosynthetic apparatus of *S. latissima* being unaffected by hyposalinity stress in this study, while further evidencing nitrogen availability as a key limiting factor in kelp photosynthetic rate.

UNDERWATER COMPANION PLANTING – SEAWEED POLYCULTURE

As the interest in seaweed cultivations rises in the West, and the range of biomolecules and bioactive compounds in seaweeds become more attractive products, the concept of having a multi-species seaweed farm seems to have good potential. In terrestrial systems this idea is known as polyculture, or companion planting, where multiple plant species are grown in the same area to conger benefits to one another and increase overall crops yields and productivity. The aim of this paper was to assess to what extent there are interactions, both synergistic and competitive, between two different kelp species and one green seaweed in polyculture. The selected species for this experiment are of the number of most widely cultivated in Europe, the two kelps, *S. latissima* and *L. digitata*, and the green seaweed *U. fenestrata*. Further, to investigate potential competition for nitrogen, the most limiting nutrient for seaweed growth, two treatments were used, either ambient nitrogen in surface seawater or artificially elevated nitrogen.

Overall, there were strong interactive effects of both culture system (companion planting vs. monoculture) and nutrient enrichment on the elemental composition of *S. latissima* and *L. digitata*, and the biomass growth of *L. digitata.* Generally, companion planting had negative effects, but nutrient enrichment neutralised these negative effects. However, despite the nullifying effect of nitrogen enrichment, companion planting can still result in negative effects due to allelopathic interactions, as in the case of the ETR of *L. digitata*.
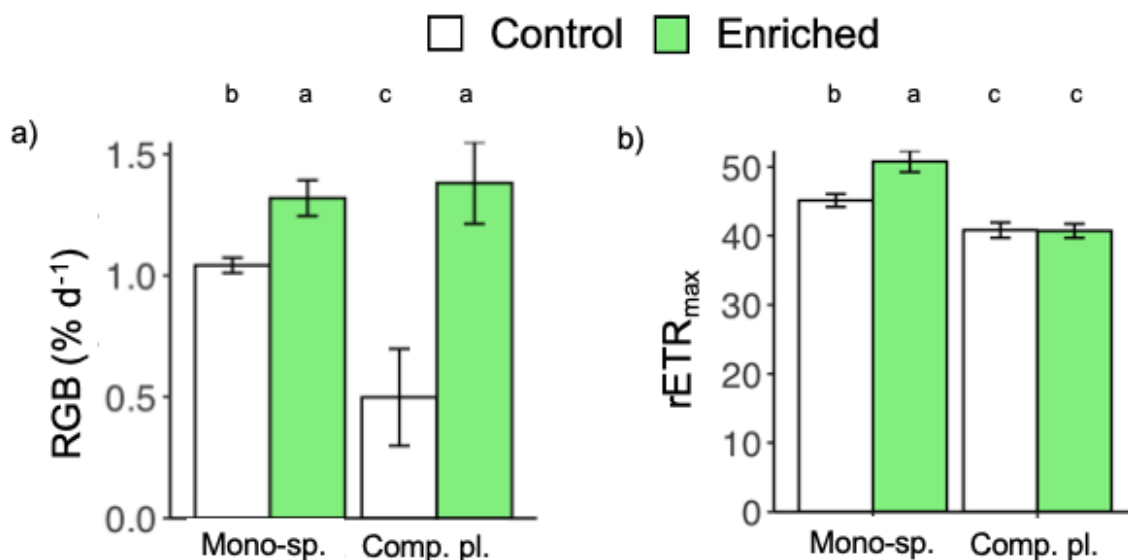

Fig. 22. The response of *Laminaria digitata* to companion planting and nutrient elevation is terms of the relative growth of biomass (RGB) and relative maximum electron transfer rate (rETRmax)

The interaction of cultivation type and nutrient level is clear in the case of *L. digitata* (Fig. 22). Under ambient levels of nitrogen there was a severe decline, by about half, in kelp biomass growth in companion planting compared to monoculture. However, when nitrogen was elevated, biomass growth in companion planting was comparative to growth in monoculture. This evidences nutrient competition as a potential problem in multi-species seaweed cultivations, where species with faster nutrient uptake dynamics outcompete slower species, in this case *L. digitata*. However, the neutralisation of the negative effects of companion planting by nitrogen did not occur with regard to ETR. In this case, ETR was elevated in monoculture with high nitrogen in comparison to monoculture at ambient nitrogen, but in both companion planting treatments ETR was significantly lower than both monoculture treatments. These results imply that the persistent negative effects of companion planting on ETR are a result of something other than nutrient competition. This could potentially be due to an allelochemical effect, where exudates from another species in the system is inhibiting *L. digitata* somehow.

However, this potential allelochemical effect was only observed to impact *L. digitata*, while the electron transfer rate of the other two species was apparently unaffected. In the cases of *S. latissima* and *U. fenestrata,* the major effect was that of apparent nutrient competition in companion planting at ambient nitrogen levels, which was nullified by the increased provision of dissolved inorganic nitrogen (Fig. 22 and 23).
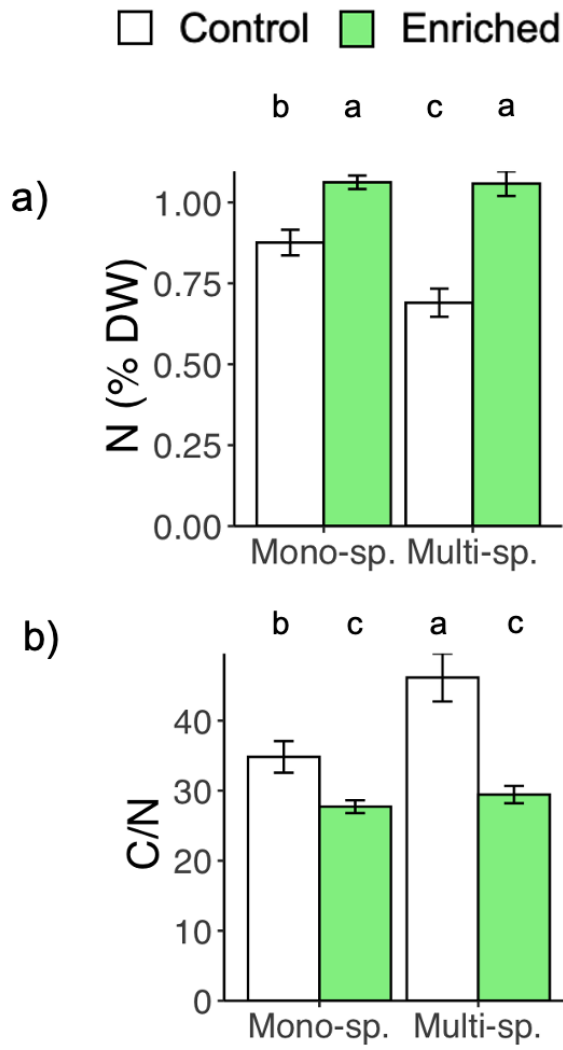
Fig. 23. The response of *Saccharina latissima* to companion planting and nutrient elevation is terms of tissue nitrogen content (N%) and carbon : nitrogen ration (C/N)

MAIN CONCLUSIONS

Overview of key conclusions

The main conclusions of the thesis in relation to the specific papers:

PAPER I: There is a strong beneficial effect of growing kelps in co-culture with filter feeding bivalves, such as the blue mussel, *Mytilus edulis,* and the Pacific oyster, *Magallana gigas.* The strongest effects in this study were related to biofiltration and fertilisation. Bivalve filtration drastically reduced the amount of particles in the seawater supplied to the seaweeds, resulting in a decline in epiphytes on the seaweed. With regard to fertilisation, both bivalve species significantly elevated nutrients, which were subsequently incorporated into kelp tissue.

PAPER II: The strong biofiltration effects observed in **PAPER I** were seen to carry over into open water co-cultivation with commercial blue mussel farms. Epiphyte coverage was reduced by around 50% on kelp grown in co-culture with blue mussels, rather than in monoculture, as a result of mussel filtration. This biofiltration effect is further evidenced by the observed increase light intensity downstream of farms. Co-culture also significantly enhanced kelp growth and yield, again as an indirect result of bivalve filtration.

PAPER III: When nitrogen levels are replete, as may be the case in IMTA systems with fish, the additive effect of mussel effluent had no significant effects on kelp growth or photosynthetic rate. However, photosynthetic pigments were significantly enhanced by bivalve effluents despite simultaneous nitrogen enrichment, indicating the continued bioremediative effect of kelp when nitrogen is non-limiting as well as an increased production of valuable bioactive compounds.

PAPER IV: On the short term, it appears that prior exposure to elevated nitrogen allows *S. latissima* to become more resilient to hyposalinity shock and facilitates a faster recovery. In terms of acclimation, some variables, such as growth, are driven primarily by salinity, whereas others, such as photosynthetic rate, were driven purely by nitrogen availability. There was also limited evidence that under suboptimal salinities *S. latissima* invests in width-wise growth and nutrient storage in anticipation of future, more optimal conditions.

PAPER V: Polyculture, or companion planting, of multiple seaweed species is largely dependent on nutrient bioavailability, particularly nitrogen. When nitrogen

was insufficient to support all three species in our experimental system there were declines in ecophysiological performance. However, when nitrogen was enriched, the negative effects of polyculture were largely neutralised. Overall, it appears that nutrient competition is a major issue in seaweed polyculture. Lastly, there was evidence for an allelochemical effect on the inhibition of photosynthesis of *L. digitata*, potentially from *U. fenestrata.* Understanding such chemically mediated competition between seaweed species will be of great importance in the development of polyculture systems.

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Big thanks to Luca, you can view this as your second PhD thesis! Apes together strong. Alice and Joel, thanks for the endless support and help along the way! I'd also like to thank my fellow PhD comrades Wouter and Sam who trod the path before me, I learned that, in essence, everything is basically just funny. Big thanks to my officemate Annelous for all the laughs, work disrupting conversations and Dutch biscuits.

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Anothai, thank you for your support and patience, particularly in the last year or so. I would probably have gone a bit mad without you!

Lastly I'd like to thank my Mum, Dad and sister Laura, as well as the rest of my family, for their love and support, and for helping me to get to this point today. It has been hard seeing so little of you all and I am sorry for being away for so long. All my love.

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