Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Sofie Uttian Alstad

Bioremediation Potential of the Green Algae *Ulva* sp. Cultivated in Wastewater from Marine Recirculating Aquaculture Systems (RAS)

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Trondheim, May 2022
Sofie Uttian Alstad

Abstract

Ulva is a widespread green algal genus with promising potential regarding uptake of nutrients from Recirculating Aquaculture Systems (RAS) wastewater because of its high tolerance to various temperatures, water qualities, nutrient levels and salinities. The uptake of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}) from wastewater, originating from RAS farming of Atlantic Salmon (*Salmo salar*) smolt and post smolt, were studied in *Ulva* sp. collected from the coast of Central Norway in April and September 2021. Two uptake experiments were conducted, both determining the initial short-term nitrogen and phosphorus uptake kinetics, by following the depletion of substrate concentrations over an incubation period of 8 and 10 hours. The uptake was either measured for nutrient concentrations in a gradient from 25 % to 100 % RAS-water, or in a biomass density gradient ranging from 0.25 g wet weight (WW) to 4 g WW per 250 mL with same RAS-water concentrations.

Ammonium uptake rates, related to dry weight biomass (DW), revealed a linear relationship with RAS-water concentration. The maximum measured ammonium uptake rate was 387 ± 18 μg g_{DW}^{-1} $hour^{-1}$ for the 100 % RAS-water treatment (~ 160 μM NH_4^+). Preferred nitrogen source in *Ulva* sp. was discovered to strongly be affected by the ratio of available nitrate and ammonium in the wastewater from RAS. At a nitrate:ammonium ratio of 12:1, ammonium was found the be the favored nitrogen source regarding uptake, and consequently inhibited the uptake of nitrate. However, at a higher nitrate:ammonium ratio (152:1), an uptake in nitrate in addition to ammonium uptake was discovered within the first 80 minutes of the experimental period, with a maximum measured nitrate uptake of 54 748 ± 7 366 μg g_{DW}^{-1} h^{-1}.

The lowest density tested for in this study (0.25 g_{WW} per 250 mL) were found to have the highest uptake rate of ammonium (82 ± 3 μg g_{DW}^{-1} h^{-1}) and nitrate (54 748 ± 7 366 μg g_{DW}^{-1} h^{-1}) related to biomass, and the uptake rate decreased negative exponentially with an increase in density. This can be explained by more nutrients being available per gram of *Ulva* sp. in the lower densities. However, the highest density (4 g_{WW} per 250 mL) depleted the nutrients from the RAS medium more rapid compared to the lower ones.

Internal tissue concentrations of carbon, nitrogen and phosphorus were revealed to not be significantly different after exposure to high nutrient medium compared to the initial concentration, indicating that tissue concentrations were a bad indicator for nutrient uptake in this current study. However, initial carbon:nitrogen ratios in the tissue varied among *Ulva* sp. harvested in April (C:N ratio of ~ 7) and September (C:N ratio of ~ 13), possibly explained by the experiments being conducted pre and post phytoplankton spring-bloom.

From the results of the current study, the opportunistic macroalgae *Ulva* sp. was recognized as a potential organism to clean wastewater and to bioremediate nutrients from low saline (~ 15 ppt) RAS water as it holds the potential to remove ammonium and nitrate. However, the water released from the biofilter should have a high nitrate:ammonium ratio to optimize the removal rate of nitrate, which is the most abundant nitrogen source in such medium after water treatment in the biofilter.

Sammendrag

Ulva er en utbredt grønnalgeslekt som på grunn av dens høye toleranse for ulike temperaturer, vannkvaliteter, næringsnivåer og saltholdigheter har et lovende potensiale når det gjelder opptak av næringsstoffer fra avfallsvann fra resirkulerende akvakultursystemer (RAS). Opptaket av ammonium (NH_4^+), nitritt (NO_2^-), nitrat (NO_3^-) og fosfat (PO_4^{3-}) fra avfallsvann, som stammer fra RAS oppdrett av atlantisk laks (*Salmo salar*) smolt og post smolt, ble studert i *Ulva* sp. høstet fra kysten av Midt-Norge i april og september 2021. Det ble gjennomført to opptaksforsøk, som begge bestemte opptaksraten av nitrogen og fosfor ved å følge reduseringen av næringskonsentrasjoner i substratet over en inkubasjonsperiode på 8 og 10 timer. Opptaket ble enten målt for næringskonsentrasjoner i en gradient fra 25 % til 100 % RAS-vann, eller i en biomasse gradient fra 0,25 g våtvekt (VV) til 4 g VV makroalge per 250 mL med samme RAS-vannkonsentrasjoner.

Opptaksraten av ammonium, relatert til tørrvekten av biomassen (TV), viste et lineært forhold med RAS-vann konsentrasjonene. Den maksimalt målte opptaksraten av ammonium var 387 ± 18 µg g_{TV}^{-1} $time^{-1}$ for 100 % RAS-vann (~ 160 µM NH_4^+). Foretrukket nitrogenkilde i *Ulva* sp. ble funnet å være sterkt påvirket av forholdet mellom tilgjengelig nitrat og ammonium i avfallsvannet fra RAS. Ved et nitrat:ammonium forhold på 12:1 ble ammonium vist å være den foretrukne nitrogenkilden, og hemmet følgelig opptak av nitrat. Ved et høyere nitrat:ammonium forhold (152:1) ble det imidlertid konstatert et opptak av nitrat i tillegg til ammoniumopptak innen de første 80 minuttene av forsøksperioden, med et maksimalt målt nitratopptak på 54 748 ± 7 366 µg g_{TV}^{-1} $time^{-1}$.

Den laveste tettheten testet for i denne studien (0,25 g VV per 250 mL) ble funnet å ha den høyeste opptaksraten av ammonium (82 ± 3 µg g_{TV}^{-1} $time^{-1}$) og nitrat (54 748 ± 7 366 µg g_{TV}^{-1} $time^{-1}$) relatert til biomasse, og opptakshastigheten sank negativt eksponentielt med en økning i tetthet. Den høyeste tettheten tok dog opp næringsstoffene fra RAS-mediet raskere sammenlignet med de lavere.

Vevskonsentrasjoner av karbon, nitrogen og fosfor ble funnet å ikke være signifikant forskjellig etter eksponering for høyt næringsmedium sammenlignet med den opprinnelige konsentrasjonen, noe som indikerer at vevskonsentrasjoner var en dårlig indikator for næringsopptak i denne studien. Imidlertid varierte karbon:nitrogen forholdene i vevet i kontroll-prøvene mellom *Ulva* sp. høstet i april (C:N-forhold på ~ 7) og september (C:N-forhold på ~ 13), muligens forklart av at eksperimentene ble utført før og etter våroppblomstringen av fytoplankton.

Fra resultatene i denne studien, kan den opportunistiske makroalgen *Ulva* sp. bli sett på som en mulig organisme til å rense avfallsvann og bioremediere næringsstoffer fra lavt saltholdig (~ 15 ppt) RAS-vann, da det har potensialet til å fjerne ammonium og nitrat. Vannet som går ut av biofilteret bør imidlertid ha et høyt nitrat:ammonium forhold for å optimalisere opptaket av nitrat, som er den mest rikelig nitrogenkilden i et slikt medium.

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Abbreviations

AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
ASW	Artificial Seawater
ATP	Adenosine triphosphate
CI	Confidence Interval
DIN	Dissolved Inorganic Nitrogen
DW	Dry wright
E1	Experiment I: RAS-water gradient
E2	Experiment II: Biomass gradient
IMTA	Integrated Multitrophic Aquaculture
N_2	Nitrogen gas
NH_4^+	Ammonium
NiR	Nitrite reductase
NO_2^-	Nitrite
NO_3^-	Nitrate
NR	Nitrate reductase
NS-EN-ISO6878	Norwegian Standard for determination of inorganic phosphorus
NS4745	Norwegian Standard for determination of inorganic nitrogen (NO_2^- /NO_3^-)
NTNU	Norwegian University of Science and Technology
PO_4^{3-}	Phosphate
RAS	Recirculating Aquaculture System
Rpm	Rounds per minute
SE	Standard Error
TAN	Total ammonium nitrogen
TBS	Trondhjem Biological Station
WW	Wet weight

1 Introduction

Aquaculture is the farming of aquatic animals and plants in either freshwater, seawater or brackish water. It is the fastest growing food production sector and is fundamental when it comes to ensuring a food secure future (Troell et al., 2009; Aich et al., 2020; FAO, 2020). By 2050, the world population is expected to exceed 9 billion people (FAO, 2020), and solutions to increase the production while keeping the industry sustainable is needed (Wik et al., 2009). Reducing the amount of wastewater discharge, improving water recycling efficiency and moving a major part of the seafood production to lower trophic levels are among the key factors contributing to a sustainable aquaculture system (Olsen, 2011; Tom et al., 2021).

Environmental concerns regarding pollution related to wastewater from aquaculture have arisen during the past years. In addition, water scarcity and depletion of natural water resources are found to be one of the foremost challenges faced by the world today. These concerns demand for sustainable technologies for treatment of wastewater from aquaculture (Tom et al., 2021). Environmental challenges related to traditional aquaculture in open cages can be avoided with land based recirculating aquaculture systems (RAS), as it provides the opportunity to have control on the water effluents as well as escapes from sea cages and parasite transmission (Shpigel & Neori, 1996; Bjørndal & Tusvik, 2019). Additionally, RAS provide opportunities to increase wastewater and sludge management, reduce the water usage and recycle the nutrients into commercial valuable biomass (Hurd et al., 2014; Ahmed & Turchini, 2021; Tom et al., 2021).

1.1 Recirculating Aquaculture Systems

Norway is the largest producer of Atlantic salmon (*Salmo salar*), and farming of smolts and post smolts on land in RAS is increasing (Dalsgaard et al., 2013). Because of their diadromous life cycle with natural migration between freshwater and marine water (Thorstad et al., 2012), the water in Recirculating Aquaculture Systems (RAS) does normally have a lower salinity compared to natural seawater. RAS are land-based, indoor farming facilities where aquatic species are stocked in tanks with a controlled water environment (Aich et al., 2020). RAS are found to be effective farming units regarding managing the volume of wastewater as toxic pollutants is removed and the treated water is recycled. Only ~ 10 % of the total water volume need to be replaced with fresh water daily, giving a recirculating degree of ~ 90 % and making it a more environmentally friendly alternative compared to traditional aquaculture farming (Ahmed & Turchini, 2021; Tom et al., 2021).

Mechanical and biological filtration methods are applied to the water before it recirculates back into the system (Ahmed & Turchini, 2021). This is schematic visualized in Figure 1.1. The water in RAS will particularly contain organic components that originates from faeces and excess feed (Neveux et al. 2017; Tom et al. 2021). In addition, the fish will excrete inorganic dissolved nutrients, such as nitrogen and phosphorus (Tom et al. 2021). The nitrogen compounds ammonia (NH_3) and ammonium (NH_4^+) are the main excretory products and occurs at the gill surface. Together, these two compounds are referred to as total ammonia nitrate (TAN) and will exist in an equilibrium that is mostly influenced by the pH. At high concentrations, TAN become toxic for the fish (Moksness et al., 2004). Hence, RAS rely on a biofiltration process to convert the toxic pollutant TAN into nitrite

(NO_2^-) and nitrate (NO_3^-) (Tom et al. 2021). This process is carried out with the use of nitrifying bacteria (Schreier et al., 2010). The nitrifying process in presented in Equation 1.1 and 1.2.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ + 4e^- \ (Nitrosomanas \text{ sp.}) \quad (1.1)$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- + 2e^- \ (Nitrobacter \text{ sp.}) \quad (1.2)$$

The recirculation of water back into the tanks offers a possibility for large scale and sustainable aquaculture production (Wik et al., 2009). However, the accumulation of NO_3^- in the wastewater serve as the major limitation. Too high levels of NO_3^- should be avoided to prevent toxic concentrations for the fish, and this is today solved by the daily water exchange. Nevertheless, other solutions are possible. One of these solutions is to apply a denitrification process in the biofilters, where the inorganic nitrogen compounds from the nitrifying process is converted to nitrogen gas (N_2) and removed from the system. This is a slow process, and a more sustainable option is to culture autotrophic organisms with use of the wastewater in an Integrated Multitrophic Aquaculture System (Tom et al. 2021).

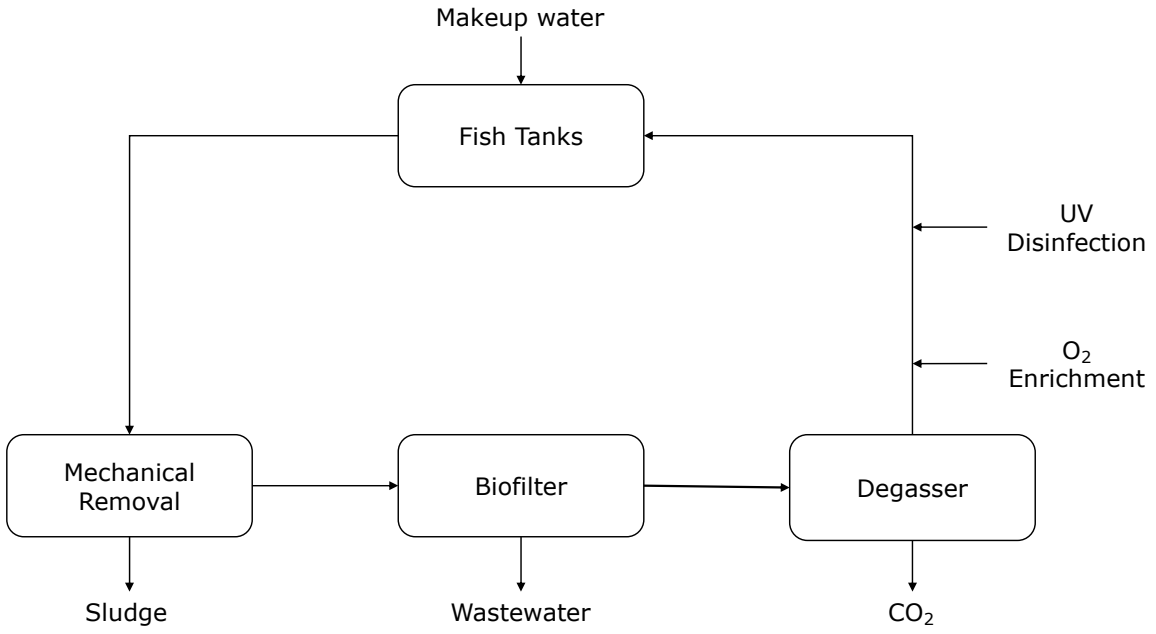

Figure 1.1: Simplified schematic overview of a Recirculating Aquaculture system (RAS). Illustration modified from Bregnballe, 2015.

1.2 Integrated Multitrophic Aquaculture Systems

Integrated Multitrophic Aquaculture (IMTA) is found to be a sustainable method to treat wastewater from aquaculture (Wang et al., 2012; Tom et al., 2021). IMTA involves the co-culturing of fed species (as finfish) with species from lower trophic levels that utilize inorganic (as macroalgae) and organic nutrients (as shellfish) for energy and growth. This results in waste or byproducts from one tropic level to become food for another trophic level (Wang et al., 2012). Thus, a minimum input cost can facilitate production of commercial products, as well as serve as a cleaning solution since nutrients is partially removed (Troell et al., 2009; Hurd et al., 2014; Tom et al., 2021).

1.2.1 IMTA with macroalgae

Several studies have demonstrated the efficiency of macroalgae on the treatment of fish effluents from aquaculture systems and shown that macroalgae are well suitable species to be cultivated in an IMTA system (e.g., Cohen & Neori, 1991; Neori et al., 2000; Wang et al., 2012; Ben-Ari et al., 2014). The produced biomass can be used for human consumption, animal feed, nutraceuticals, bioenergy, or fertilizer (Neveux et al., 2017). Macroalgae has for a long period been utilized as a feed resource for molluscs and using it as feed ingredients in aquaculture is attractive and implies that the sector is moving towards being self-sustaining (Neori et al., 2000; Olsen, 2011). Beside the direct contribution to produced biomass, macroalgae can extract inorganic nutrients (nitrogen and phosphorus) from surrounding waters, which makes integration of macroalgae cultivation with the farming of animal species an interesting production system (FAO, 2021).

RAS has the possibility to be incorporated into an IMTA system. Here, macroalgae can be used to bioremediate the excess nitrogen from the RAS water (Ahmed & Turchini, 2021). Bioremediation is described in Neveux et al. (2017) as «the use of biological organisms under controlled conditions to degrade, neutralize and/or remove harmful contaminants from polluted site». The optimal macroalgae species to be cultivated in RAS will be one having tolerance of high nitrogen concentrations and have high nitrogen uptake rates. In addition, it should have tolerance for lower salinities compared to natural seawater, high growth rates and capacity to store nitrogen and phosphorus in the tissue. Lastly, a potential market value would be optimal (Hurd et al., 2014).

1.3 Macroalgae

Macroalgae, also known as seaweed, are macroscopic and multicellular organisms divided into groups based on their main pigmentation: red (Rhodophyta), brown (Phaeophyta) and green (Chlorophyta) algae. Light serves as their foremost source of energy through photosynthesis. Hence, macroalgae live between the top of the intertidal zone and the maximum depth to which enough light for growth is available. In addition, temperature, salinity, water motion and nutrient availability are abiotic factors affecting seaweed growth and their physiological state (Hurd et al., 2014; Postma et al., 2017).

1.3.1 Nutrient uptake and assimilation kinetics

Different inorganic nutrients have different uptake mechanisms and the process to take up and assimilate diverse sources of nutrients vary (Harrison & Hurd, 2001). Hurd et al. (2014) define uptake as the transport across the cell membrane of macroalgae while assimilation is described as the reactions taking place in combining inorganic ions into organic molecules, such as amino acids. Incorporation is a term defined as processes that unite these organic molecules into macromolecules such as proteins and nucleic acids.

Dissolved inorganic nitrogen (DIN) functions as an important compound in biomolecules and is the main limiting nutrient for macroalgae growth, especially during the summer months when phytoplankton blooms have depleted the nitrogen concentration substantially compared to the winter months (Hanisak, 1983; Troell et al., 2009; Hurd et al., 2014; Ibrahim et al., 2014). No macroalgae are known to fix nitrogen from nitrogen gas (N_2), thus the available inorganic sources for uptake are nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) (Hurd et al. 2014). NO_3^- and NO_2^- are believed to require active transport to cross the cellular membrane as they are naturally and typically found in the micromolar range in the external seawater and in the millimolar range within the seaweed. In addition, nitrate can be stored inside the macroalgae cells at concentrations exceeding substrate concentrations (Harrison & Druehl, 1982; Harrison & Hurd, 2001). NO_3^- can be reduced to NO_2^- or be incorporated in the cytoplasm and vacuoles in intracellular pools (I-DIN). The reduction of NO_3^- to NO_2^- is catalyzed by the enzyme nitrate reductase (NR), while the reduction of NO_2^- to NH_4^+ is catalyzed by nitrite reductase (NiR) (Berges, 1997; Shpigel et al., 2019). NiR is usually more active than NR, suggesting that the limiting step in reduction of NO_3^- to NH_4^+ is reduction by NR (Hurd et al. 2014). Storage of NO_3^- in vacuoles may occur when the uptake rate of NO_3^- is greater that the conversion rate to NO_2^-. Further, NO_2^- is reduced to NH_4^+ in the chloroplast. Here, NH_4^+ is assimilated into biomolecules as amino acids (Harrison & Hurd, 2001). This is schematic visualized in Figure 1.2.

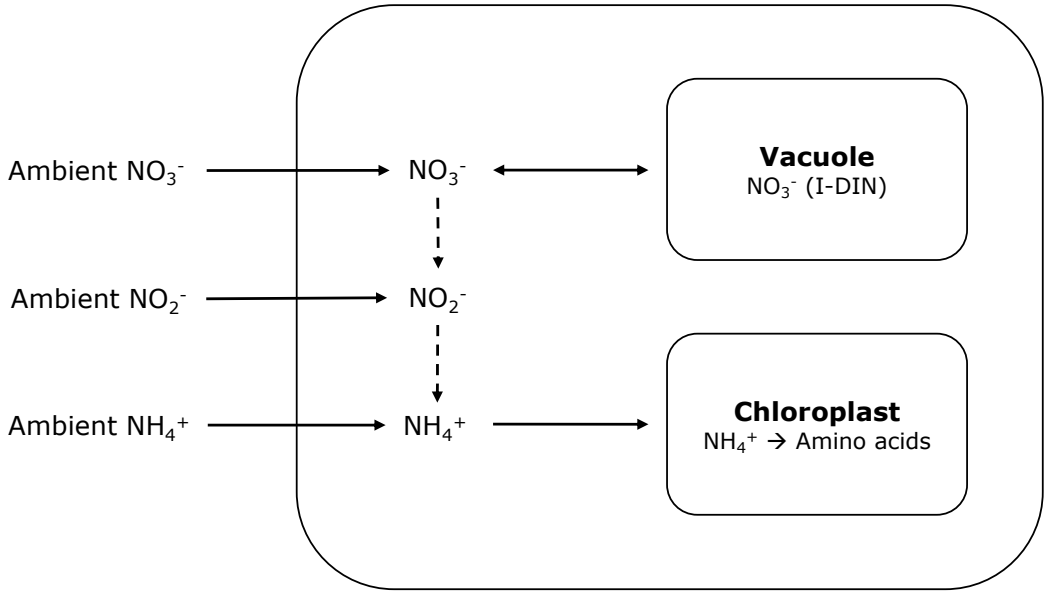

Figure 1.2: Simplified schematic overview of nitrogen uptake in an algal cell. Illustration from Forbord (2020).

There have only been a few studies on phosphorus (P, mainly available as the inorganic ions PO_4^{3-} and $H_2PO_4^-$) uptake kinetics in seaweed, but a preliminary study indicate that P is actively taken up (Lavery & McComb, 1991). Phosphorus is important regarding energy transfer (ATP), proteins, phospholipids and nucleic acids. Generally, it is not the main limiting nutrient for growth of algae in the Norwegian coastal waters (Hurd et al., 2014; Svåsand et al., 2017).

Uptake of ions that require an active uptake process may be saturated with increasing substrate concentration (Figure 1.3 B). The uptake rate can in such case be described by a rectangular hyperbola when uptake rate (V) and substrate concentrations (S) are plotted against each other. This is further described by the Michaelis-Menten Equation (1.3) which includes the maximum uptake rate (V_{max}) and the half-saturation value (K_s). K_s is the substrate concentration at ½ V_{max} (Rees, 2003; Hanisak, 1983; Hurd et al., 2014).

$$V = V_{max} \frac{S}{K_S + S} \tag{1.3}$$

If transport occurs by passive diffusion, the transport rate will be directly proportional with the external concentration (Figure 1.3 A) (Taylor et al., 1998; Philips & Hurd, 2004; Hurd et al., 2014). NH_4^+ can be taken up through passive transport, and directly converted into amino acids in the chloroplast (Figure 1.2) (Syrett, 1981). Hence, unsaturated uptake is common for NH_4^+ (Phillips & Hurd, 2004; Abreu et al., 2011; Martínez et al., 2012), but it has also been described for NO_3^- (Harrison et al., 1986; Ahn et al., 1998; Sánchez-Barredo et al., 2011; Martínez et al., 2012).

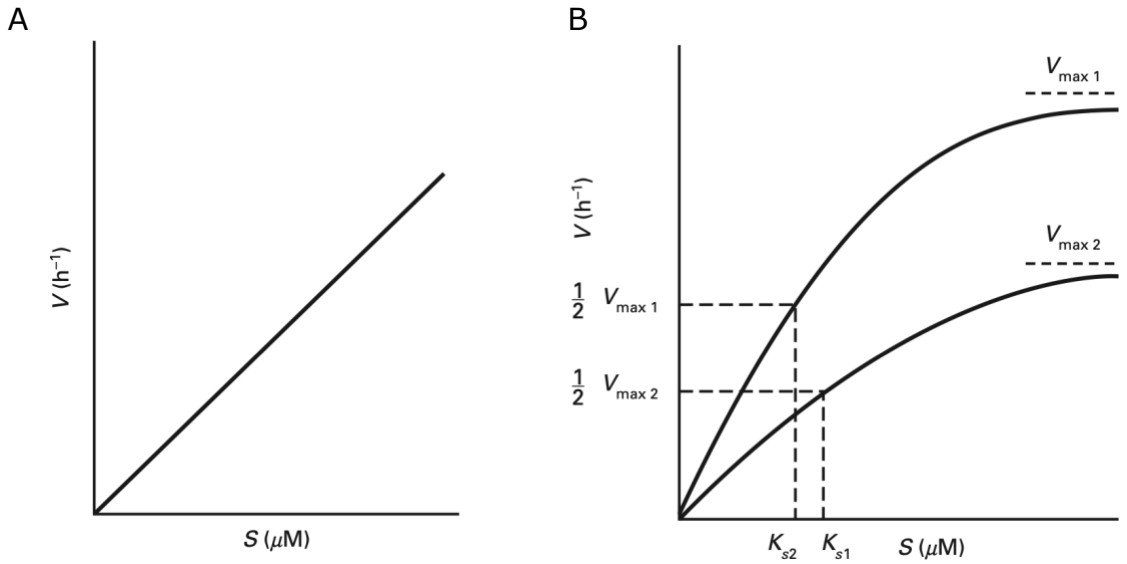

Figure 1.3: Hypothetical plots of nutrient uptake rates (V) and concentrations of the limiting nutrients (S) for A) passive diffusion and B) facilitated diffusion or active transport, where $V_{max\ 2}$ = ½ $V_{max\ 1}$ and consequently $K_{s1} < K_{s2}$. Figures from Hurd et al. (2014).

Uptake characteristics can also be related to the species nutritional history, where nutrient limited macroalgae exposed to high substrate concentrations may experience a three-phased uptake (Pedersen, 1994). Initially, a rapid «surge» uptake will fill the internal storages of the algae. Then, internal uptake mechanisms will limit the uptake. Lastly, reduced nutrients in the external substrate are becoming the limiting factor and hence reduced uptake will occur. In addition, macroalgae species internal storage capability of nutrients usually correlates with its environmental distribution. Here, low storage capacity is typically related to species adapted to eutrophic environments, while high storage capacity often is linked to species living at nutrient limited sites, making them able to sustain growth for longer periods (Fujita, 1985; Hurd et al., 2014).

1.3.2 *Ulva* sp. and its bioremediation potential

The annual bloom forming species of the genus *Ulva* (Chlorophyta) (Nielsen et al., 2012; Hurd et al., 2014) is a cosmopolitan and opportunistic green macroalgal species (Littler & Littler, 1980; Hurd et al., 2014; Obolski et al., 2022). They have a diplohaplontic sexual cycle, with isomorphic life stages (Alström-Rapaport, 2010; Hurd et al., 2014), which results in similar morphology between the gametophyte and sporophyte. The species is distributed in many different ecological conditions (Littler & Littler, 1980; Hurd et al., 2014) and are during spring and summer seasons dominant along marine coasts (Ogawa et al., 2013). They are common throughout the world in both marine and estuarine habitats, making them present in various temperatures, water qualities, nutrient levels and salinities (Tan et al., 1999; Obolski et al., 2022). *Ulva* sp. can naturally be found in salinity ranges of 0.5-49 ppt, but *Ulva* species with leaf-shaped thalli (as the one used in this study) are normally not found below salinities of 10 ppt (Rybak, 2018).

Ulva species are usually grouped by the external morphology of their thallus, being either tubular, leaf-like or tubular leaf-like (Rybak, 2018). For species in the leaf like functional group (e.g., *Ulva fenestrata*, Figure 1.4), their thallus is composed of two cell layers and

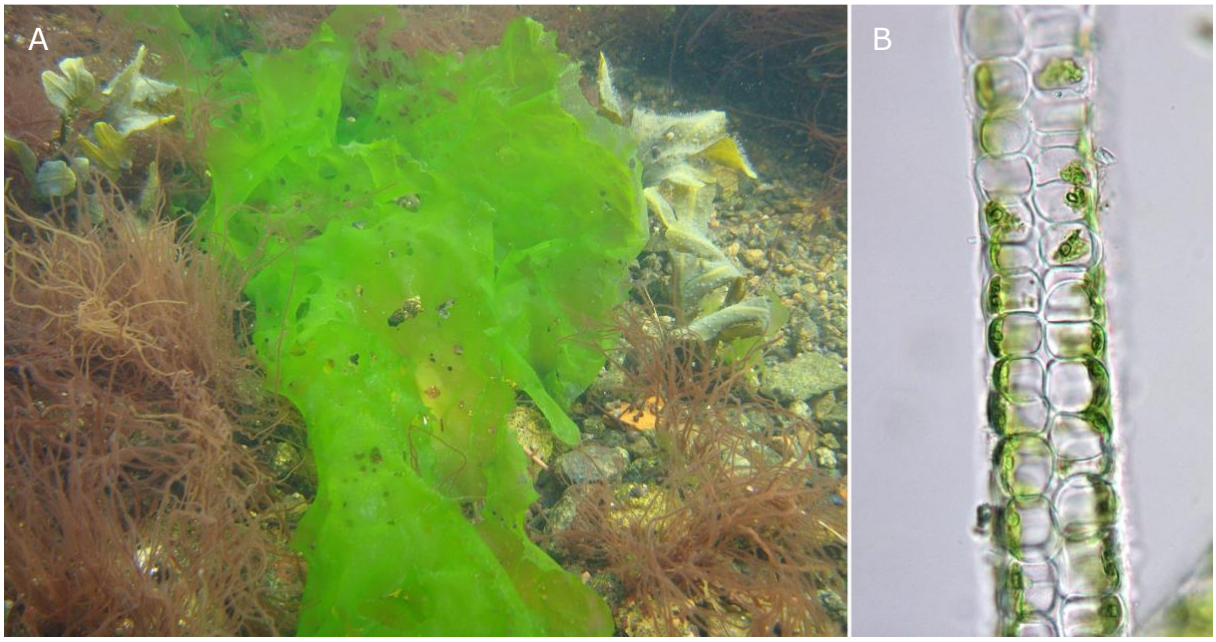

Figure 1.4: Sea Lettuce (*Ulva fenestrata*). a) Field photo. b) Cross section showing that the thallus is two cell layers thick (Photos: Rueness & Nøkling-Eide, 2021).

are thus having all its cells in contact with the water (Ale et al., 2011; Breure, 2014; Lubsch & Timmermanns, 2018). This gives the species a high surface:ares ratio (SA:V), which often feature rapid nutrient uptake (Rosenberg & Ramus, 1984; Hein et al., 1995; Neori et al., 2004). This suggests that they in addition to high nutrient uptake also are predicted to have high rates of growth and photosynthesis (Hurd et al., 2014).

Ulva sp. has consequently been identified as an ideal candidate to function as a biofilter (Ben-Ari et al., 2014; Shpigel et al., 2019) and to bioremediate nutrients from aquaculture wastewater (Cohen & Neori, 1991; Nielsen et al., 2012). Despite a current lack of established uses for the *Ulva* sp. produced, its research for potential use in human food and animal feed is increasing (Neveux et al., 2017).

Because of *Ulva* species' high plasticity and simple morphologies, they can be hard to identify (Ogawa et al., 2013). Linné described *Ulva* sp. found in Scandinavia as *Ulva lactuca.* However, recent molecular analysis (2019) found the species living across northern coasts (as the coast of Norway) to be *Ulva fenestrata.* In this thesis there is chosen to only use the genus *Ulva* sp., commonly known as sea lettuces (Rueness & Nøkling-Eide, 2021).

1.4 Aim of study

The overall aim of this study was to characterize the bioremediation potential of *Ulva* sp. in RAS water, by investigating the uptake rates of nutrients in the wastewater, being ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}). Uptake rates of nitrogen and phosphorous in the macroalgae were characterized for different substrate concentrations (RAS water) and at different biomass densities. These uptake rates will give information of the bioremediation potential of *Ulva* sp., and further gain knowledge on sustainable cleaning solutions for RAS water.

To characterize the bioremediation potential, two separate uptake experiments were conducted:

I. A gradient in RAS water concentration to investigate the uptake rate across low to high RAS water concentrations, where the biomass was kept constant.

II. A macroalgae density-gradient study to investigate the uptake rate across low to high biomass-density, where the RAS water concentration was kept constant.

Uptake rates were determined by following the depletion of substrate concentrations over an incubation period of 8 and 10 hours. Two hypotheses were formulated:

H1. *Ulva* sp. will favor ammonium (NH_4^+) as nitrogen source over nitrate (NO_3^-).

H2. The ammonium (NH_4^+) uptake rate will increase linearly with increased exposure concentration.

2 Materials and methods

2.1 Collection of RAS water and macroalgae

Individuals of *Ulva* sp. were provided by Statsnail AS in April and September 2021, collected from Oksvoll, Ørland in Central Norway (63°48´30.1"N, 9°35´51.3"E). Wastewater from fish RAS were delivered from two different facilities, both farming Atlantic Salmon (*Salmo salar*). Characteristics of the RAS water are presented in Table 3.1. Due to that one facility was farming post smolts and the other farming smolts, the salinity of the RAS-waters was different, being 21 and 2.4 ppt respectively. In order to use same salinity in the two experiments, the salinity was adjusted by adding salt (Red Sea Salt) to the RAS water for the second experiment.

2.2 Study of initial ammonium, nitrite, nitrate and phosphate uptake

The uptake-experiments were conducted in a climate-regulated room at Trondhjem Biological Station (TBS, Heggdalen, Trondheim) in April and September 2021. The room temperature was kept at 13°C during the experimental processes (similar to the temperature in the fish tanks), and all water used in the experiments was acclimatized at a minimum of 12 hours before use. Until the start of the experiments, the macroalgae was kept in tanks at SINTEF SeaLab (Brattøra, Trondheim) with nutrient rich deep sea water (70 meter depth) in a flow through system for up to 8 days prior to the experiment.

2.2.1 Experimental setup and procedure

A. Experiment I – RAS-water gradient

Five RAS water concentration treatments (100 %, 75 %, 50 %, 25 % and sea water) were run with four replicates. In addition, there were two controls per treatment and two artificial seawater controls to monitor potential changes in nitrogen and phosphorus during the experiments due to microbiological activity. Setup of Experiment I can be investigated in Table 2.1 and Figure 2.1 A. The beakers were placed on an Orbital shaker (100 rpm, Orbitron M 850 x 470 mm, Infors AG, Bottmingen) to ensure water movement and homogeneous distribution of particles and molecules. To ensure photosynthetic activity (Forbord et al., 2021), a light source was placed behind the stirring table, ranging from ~ 45-70 µmol*PAR*$s^{-1}m^{-2}$ after having the beakers set up on the table.

The 100 % RAS water equals water directly from the fish farm with no adjustment. For the other gradients, there were added artificial seawater (ASW) with a salinity same as the RAS water to create the target concentration. ASW was produced by a modified composition of salts dissolved in distilled water as described in Kester et al. (1967) for a salinity of 15 ppt, presented in Table 2.2. The RAS water was initially thought to be 15 ppt, but data from the supplier revealed a higher salinity (21 ppt). RAS water used in Experiment I is therefore suggested to be in the range of 15-21 ppt. For the seawater treatments, filtrated deep sea water was used.

Table 2.1: Setup of Experiment I. RAS-water (%) and algae biomass (g_{WW}) in each bottle are given in addition to number of replicates and controls.

Experiment I (April 2021)			
% RAS-water	Biomass (g_{WW})	Replicates	Controls (without biomass)
100	1.3	4	2
75	1.3	4	2
50	1.3	4	2
25	1.3	4	2
0 (Sea water)	1.3	4	2
0 (Artificial Sea water)	-	-	2

Table 2.2: Artificial seawater constituents for a salinity of 15 ppt, modified after Kester et al. (1967).

Compound	Concentration (g L^{-1})	Compound	Concentration (g L^{-1})
NaCl	2.0	$NaHCO_3$	0.196
$MgCl_2$	5.079	KBr	0.098
$NaSO_4$	3.994	H_3BO_3	0.027
$CaCl_2$	1.123	$SrCl_2$	0.024
KCl	0.667	NaF	0.003

Macroalgae of approximately same weight (1.3 g_{WW}) were placed in each treatment bottle. The experiment started when the seaweed was added to their designed beaker and had a duration of 480 minutes (8 hours).

B. Experiment II – Biomass-gradient

Five biomass-gradients (0.25, 0.5, 1.3, 2.0 and 4.0 g_{WW} per 250 mL) were exact weighted out and the weight was recorded. The experimental setup consisted of 22 Erlenmeyer beakers (250 mL, 5 treatments, 4 replicates, 2 controls) (Table 2.3 and Figure 2.1 B). Fewer controls were needed in the second experiment due to all biomass-treatments having the same substrate concentration. In this experiment as well, the beakers were placed on an Orbital shaker (100 rpm, Orbitron M 850x470 mm, Infors AG, Bottmingen) with a light source behind, ranging from ~ 50-80 µmol*PAR*$s^{-1}m^{-2}$ after having the beakers set up on the table.

The experiment started when the algae were added to their designed beaker – all filled with RAS-water from smolt facility without modification.

All equipment used in both experiments were either acid-washed or of disposable material to avoid phosphorus contamination.

Table 2.3: Setup of uptake study II. RAS-water (%) and algae biomass (g_{WW}) in each bottle are given in addition to number of replicates and controls.

Uptake study II (September 2021)			
% RAS-water	Biomass (g_{WW})	Replicates	Controls (without biomass)
100	0.25	4	
100	0.5	4	
100	1.3	4	2
100	2	4	
100	4	4	

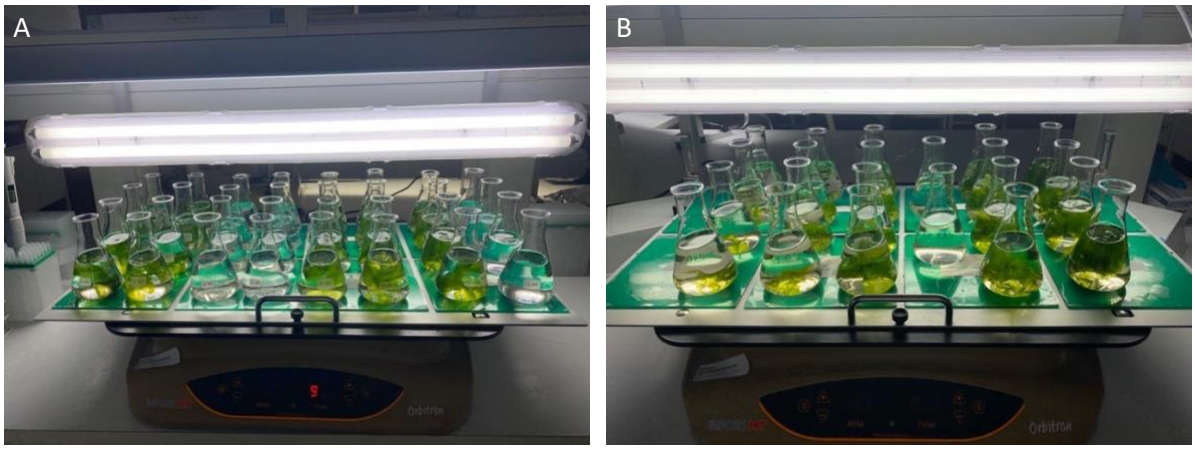

Figure 2.1: Photos of experimental setup for determination of nutrient uptake rates in *Ulva* sp. from marine salmon Recirculating Aquaculture Systems (RAS) wastewater. A) Experiment I: increasing RAS water concentrations and B) Experiment II: increasing biomass density. Photos: Sofie Uttian Alstad.

2.2.2 Sample collection

A. Water samples

4 mL water samples were collected for nutrient analysis from each beaker using a pipette after 10, 20, 40, 80, 160, 320 and 480 minutes of macroalgae incubation for uptake study I and 10, 20, 40, 80, 160, 320, 480 and 600 minutes of incubation for uptake study II (Table 2.4). This gave a total of 28 mL and 32 mL water reduction in the beakers, equivalent to 11 % and 13 % reduction in water volume for experiment I and II respectively. Samples from the control beakers were taken at 0 minutes and the last sampling point, 480 and 600 minutes for Experiment I and II, respectively. All samples from the treatment beakers were filtrated using a 0.45 µm syringe filter (VWR international, USA) to remove algal debris and transferred to pre-marked 15 mL plastic tubes before frozen until analyses (Forbord et al., 2021), that is further described in chapter 2.3.1.

The reason for running the second uptake experiment two hours longer was due to results obtained in Experiment I, showing that all nutrients were not taken up by the algae at the end timepoint. Hence, it was decided to run the second experiment two hours longer to see what occurred during the next hours.

Table 2.4: Overview of the time points for water sampling.

	Time points (min) for replicates	Time points (min) for controls
Experiment I: Sampling (4 mL)	10 20 40 80 160 320 480	0 480
Experiment II: Sampling (4 mL)	10 20 40 80 160 320 480 600	0 600

B. Tissue samples

At the end of the uptake experiments, the seaweed from all the treatment beakers were gently squeezed dry and put into pre-marked plastic bags and frozen until tissue carbon, nitrogen and phosphorus analysis.

2.3 Analyses

2.3.1 Water sample analysis of NH_4^+, NO_3^-, NO_2^- and PO_4^{3-}

The water samples were thawed at room temperature. Analysis was done photometrically on an autoanalyzer (Flow Solution IV System, O.I. Analytical), following Norwegian Standard NS4745 (1991) and NS-EN ISO 6878 (2004) for determination of NO_2^-/NO_3^- and PO_4^{3-} respectively. Analysis of NH_4^+ was performed as described in Kérouel and Aminot (1997). As the autoanalyzer can handle a maximum concentration of every compound within the standard curve, some samples needed to be diluted before analysis. The amount of dilution was based on the water environmental variables given by the suppliers (now presented in Table 3.1), yet some samples were diluted twice.

2.3.2 Tissue sample analysis: CNP elemental analysis

A. Freeze drying

Tissue samples from the treatment bottles from both experiment I and II in addition to control samples (n = 3 x 2 experiments) were freeze dried (CHRIST, Beta 1-8 LSCbasic). A preliminary test on algae of high weight was performed to estimate the amount of time needed to freeze dry the algae and was set to run for 24 hours. The samples were placed in separate glass containers or in aluminum foil placed on the shelves within the drying chamber of the freeze dryer. Following, the samples were flash frozen at -80 °C for 1 hour and freeze dried at ~ -45°C for 4 hours and ~ -60°C for the last 19 hours. Freeze dried samples were crushed into powder straight after they were taken out of the freeze dryer using a mortar and pestle. The powder was stored in plastic tubes and frozen until elemental analysis.

B. Tissue nitrogen and carbon content

The analysis of internal nitrogen and carbon content was performed by CN-analysis. Freeze dried algae samples of 1-2 mg were weighted into tin capsules while the exact weight was recorded. The capsules containing algae powder were then folded into small balls on a carbon free metallic plated using a tweezer before stored separately in 96-well plates and kept in a desiccator to prevent a humid environment. The 96-well plates containing the

sample capsules were dried in a heating cabinet at 60°C for 24 hours prior to analysis. Determination of carbon and nitrogen content in each capsule was then performed on an elemental analyzer (Vario EL cube, ELEMENTAR) with the use of acetanilide as standard solution.

Estimates of internal nitrogen and carbon content in each sample were given as a measure of µg per capsule from the elemental analyzer. This was converted to tissue content, described in Equation 2.1 for nitrogen content,

$$\mathrm{TC}_N\ (\mu g_N * \mathrm{mg}_{\mathrm{DW}}^{-1}) = \frac{\mu g_N}{\mathrm{SW}\ (\mathrm{mg}_{\mathrm{DW}})} \qquad (2.1)$$

where μg_N is the nitrogen in each capsule, SW is the sample weight recorded in each capsule (mg_{DW}) and TC_N is the tissue content ($\mu g_N\ mg_{DW}^{-1}$). The same formula was used for converting the carbon content in each sample to tissue content.

C. Tissue phosphorus content

The analysis of internal phosphorus content was performed by weighting out ~0.5 mg of freeze dried algae into a small container and transferred to pre-marked plastic bottles. The exact weight was recorded, and sample bottles were stored frozen. Distilled water (H_2O, 10.0 mL), sulfuric acid (H_2SO_4, 0.1 mL) and potassium persulfate as an oxidizing reagent ($K_2S_2O_8$, 2.0 mL) were added to each sample bottle. Then they were autoclaved at 120°C for 30 minutes to convert particulate phosphorus to inorganic phosphorus (phosphate). Thereafter, when cooled to room temperature, the samples were analyzed photometrically on an autoanalyzer (Flow Solution IV System, O.I. Analytical).

Estimates of internal phosphorus content in each sample was given as a measure of µg L^{-1} from the autoanalyzer. This was converted to tissue content, described in Equation 2.2,

$$\mathrm{TC}_P = \frac{\mathrm{P}\ * V}{\mathrm{SW}} \qquad (2.2)$$

where P is the phosphorus content in the water sample ($\mu g_P\ L^{-1}$), V is the solution volume (0.0121 L), SW is the sample weight recorded in each plastic bottle (mg_{DW}) and TC_P is the tissue content ($\mu g_P\ mg_{DW}^{-1}$).

2.4 Calculations

2.4.1 Calculation of uptake rates

Measurements of NH_4, NO_3^-, NO_2^- and PO_4^{3-} concentrations (µg L^{-1}) at each sampling point were used to follow the change in nutrient concentration in the water over time. Concentrations were related to biomass in each bottle and subsequently used in the calculation of uptake rates for each nutrient. The uptake rates were estimated in each replicate bottle according to Equation 2.3,

$$V = \frac{(S_i - S_f) * vol}{t * DW} \quad (2.3)$$

where V is the specific uptake rate (µg g^{-1} DW h^{-1}), S_i is the initial substrate concentration (µg L^{-1}), S_f is the final substrate concentration (µg L^{-1}), vol is the volume in the bottles (L), t is the time between start and end of sampling (hour) and DW is the dry weight of the total biomass in the flask (g) (Forbord et al., 2021).

2.4.2 Wet weight (WW) / Dry weight (DW)

20 separate individuals of algae (that was not a part of the uptake experiments) were weighted out and dried in a heating cabinet for 24 hours at 60°C. Their dry weight was recorded and a mean percentage of dry weight from their wet weight was established. This was used to estimate the dry weight of algae used in the treatment bottles based on their registered wet weight.

For Experiment I conducted in April 2021 a factor of 13.58 % DW of WW was established, and a factor of 22.34 % DW of WW was established for Experiment II conducted in September 2021.

2.4.3 % CNP of dry weight and tissue ratios

Results from the elemental analysis in tissue samples were chosen to be presented as percentage nutrient of dry weight (% DW). The conversion from µg mg_{DW}^{-1} to % DW is described in Equation 2.4 and 2.5,

$$TC\ (\mu g\ *\ mg_{DW}^{-1}) * 0.001 = TC\ (mg\ *\ mg_{DW}^{-1}) \quad (2.4)$$

$$\frac{TC\ (mg\ *\ mg_{DW}^{-1}) * 100}{SW\ \ (mg_{DW})} = \%\ DW \quad (2.5)$$

where TC is the tissue content of C, N or P (µg mg_{DW}^{-1}), SW is the sample weight (mg_{DW}) and 100 is the conversion factor to percentage.

Ratios of C, N and P in the algae were calculated using the formula described in Equation 2.6.

$$\frac{TC_C\ (\mu g_C\ *\ mg_{DW}^{-1})}{TC_N\ (\mu g_N\ *\ mg_{DW}^{-1})} = C{:}N\ ratio \quad (2.6)$$

2.5 Data treatment and statistical analysis

The statistical analysis and visual presentation of data was performed in R Studio (Version 1.2.5033) after treating the raw data in Microsoft Excel. Large outlying datapoints of substrate concentrations were removed based on visual inspection of the raw data set. Outliers is likely to represent contamination or air bubbles during the water sample analysis in the autosampler. This applied in Experiment I for 1 replicate at timepoint 320 minutes in treatment 100 % RAS water, 3 replicates at timepoint 320 minutes in treatment 75 % RAS water, 1 replicate at timepoint 10 at 25 % RAS-water and 1 replicate at timepoint 10 treatment Seawater for Experiment I. In Experiment II this applied for 1 replicate at timepoint 80 minutes in treatment 2 $g_{ww.}$

Linear regression analysis and analysis of variance (one-way and two-way ANOVA) combined with Post Hoc Tukey's test (95 % Confidence Interval) were used to investigate the correlation between following measurements for both Experiment I and II:

1. Reduction in nutrient concentrations of ammonium, nitrate, nitrite and phosphate in the water substrate compared to timepoints and treatments (RAS-water concentrations and biomass-density).

   The relationship between nutrient concentration in the water and time were investigated using a linear model with interaction effect between time and treatment (Nutrient concentration ~ Time * Treatment). Thus, high R^2-values and low p-values ($p < 0.05$) indicates a relationship between nutrient concentration and time, while low R^2-values and high p-values indicates no interaction between nutrient concentration and time.

2. Uptake rates (V) of ammonium, nitrite, nitrate and phosphorus compared between different RAS-water concentrations and biomass densities.

3. Differences in internal tissue carbon, nitrogen and phosphorus concentrations between treatments and compared to initial concentration (controls).

Substrate concentrations, uptake rates and internal carbon, nitrogen and phosphorus concentrations did throughout the analysis serve as response variables. Timepoints and treatments functioned respectively as continuous and categorical predictor variables. However, treatment was operated as continuous variable when linear regression coefficient (slope), R^2 values and p-values wanted to be obtained in uptake rates investigations.

In the analysis, a significance level of $p < 0.05$ was used as a standard but p-values < 0.001 will be given. All models used were confirmed normally distributed based on residual distributions and Shapiros normality tests. Data in this thesis is presented as mean ± standard error (SE). Choice of model was based on Akaike Information Criterion (AIC) and R^2-values.

3 Results

The initial nutrient concentrations (µg L^{-1}) in the two RAS waters used in Experiment I and II are expressed in Table 3.1. RAS water delivered from the post smolt facility (Experiment I) had a higher ammonium (NH_4^+) concentration compared to the RAS water delivered from the smolt facility (Experiment II). Also, water used in Experiment I had generally higher nitrite (NO_2^-) and phosphate (PO_4^{3-}) concentrations. Regarding nitrate (NO_3^-) concentrations, this was higher in Experiment II, indicating a more efficient biofilter at the RAS facility. Nutrient concentrations for each RAS water gradient in Experiment I can be investigated in Table A.1 (Appendix A). No significant changes in substrate concentrations for control samples without *Ulva* sp. were found between start and end of the experiments.

3.1 RAS-water gradient (Experiment I)

3.1.1 Substrate concentrations versus time

Nutrient concentrations (µg g_{DW}^{-1} L^{-1}) in the water for the different treatments (Seawater, 25 %, 50 %, 75 % and 100 % RAS-water) over an eight-hour time period (480 minutes) is shown in Figure 3.1. The percentage decrease in nutrients in the water from start (0 minutes) to end (480 minutes) can be investigated in Table A.2 (Appendix A).

A. Ammonium (NH_4^+)

The reduction of ammonium found in the experimental period revealed approximately a constant uptake rate in *Ulva* sp. for each RAS water concentration, but no uptake for seawater. More than 96 % of ammonium in the water was removed during the experimental period for all the RAS-water treatments (Table A.2, Appendix A). Linear regression showed a significant ($p < 0.001$) relationship between ammonium concentration in the water and time for all treatments, except for the seawater treatment ($p > 0.05$). The linear regression coefficient (slope), R^2 values and p-values are presented in Table 3.2. Also, treatments 25-50 % and 50-75 % did not significantly differ in ammonium concentration compared to each other (One-way ANOVA, Post Hoc Tukey's test). 100 % RAS-water were found to have the steepest decrease in ammonium, and the slopes decreased with lower ammonium available (Figure 3.1 A and Table 3.2).

Table 3.1: Mean ± SE nutrient values for null samples from Experiment I with RAS gradients and seawater (E1) and Experiment II with biomass gradients (E2). Wastewater used in E1 derives from a postsmolt facility, and water used in E2 derives from smolt facility.

Nutrient	Seawater E1	RAS E1 (Post smolt)	RAS E2 (Smolt)
Ammonium (NH_4^+)			
µg L^{-1}	8.1 ± 0.1	2 213.8 ± 6.7	220.0 ±8.5
µM	0.6 ± 0.0	158.1 ± 0.5	16.4 ± 0.6
Nitrite (NO_2^-)			
µg L^{-1}	1.2 ± 0.1	313.4 ± 2.7	8.9 ± 0.3
µM	0.1 ± 0.0	22.4 ± 0.2	0.6 ± 0.0
Nitrate (NO_3^-)			
µg L^{-1}	158.0 ± 0.4	27 239.2 ± 1052.1	35 013.1 ± 1476.3
µM	11.3 ± 0.0	1 945.7 ± 75.2	2 500.9 ± 105.5
Phosphate (PO_4^{3-})			
µg L^{-1}	24.9 ± 0.2	1 940.8 ± 129.7	1 411.4 ± 20.3
µM	0.8 ± 0.0	62.7 ± 4.2	45.6 ± 0.7

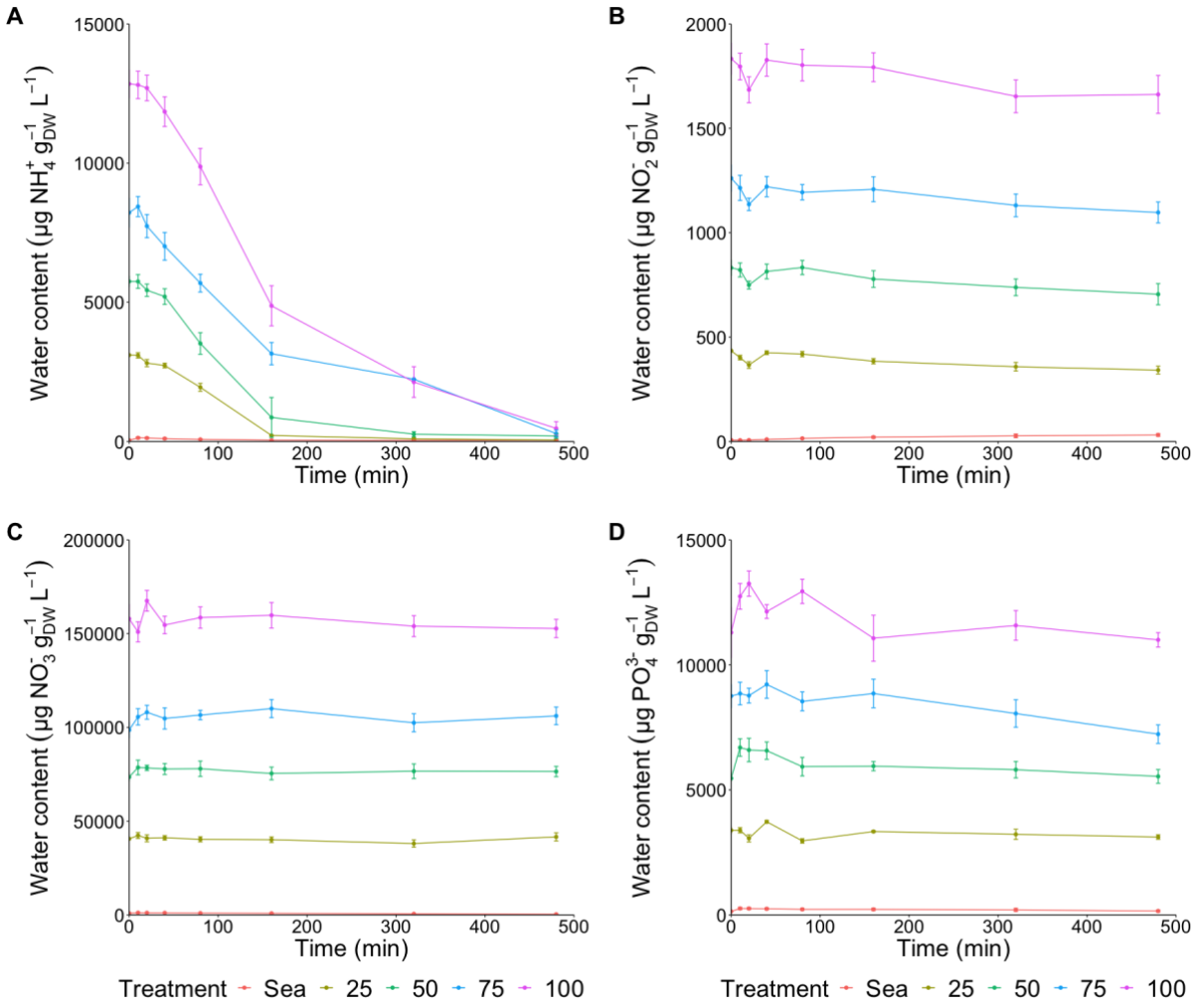

Figure 3.1: Water content concentrations (µg g_{DW}^{-1} L^{-1}) of A) Ammonium, B) Nitrite, C) Nitrate and D) Phosphate for each treatment (Seawater and 25 %, 50 %, 75 % and 100 % RAS) at different timepoints (minutes) expressed at mean ± SE (n = 4). Notice the different values on the y-axis.

B. Nitrite (NO_2^-)

Time was shown to influence the nitrite concentration for treatment 100 % and seawater only ($p < 0.001$ and $p < 0.05$ respectively, Table 3.2). For seawater, time was positively correlated with nitrite concentration, indicating that the concentration increased over time (slope = 0.1), while the nitrite concentration in 100 % RAS-water had a small decrease over time (slope = -0.3). All the treatments were found to significantly differ in nitrite concentration from each other (One-way ANOVA, Post Hoc Tukey's test). A dip in substrate concentration was also observed for every RAS-treatments at timepoint 40 minutes (Figure 3.1 B).

C. Nitrate (NO_3^-)

In every RAS-water concentration, time was found to have no significant effect on nitrate concentration in the water, suggesting no uptake throughout the experimental period, explaining the straight lines in Figure 3.1 C.

Table 3.2: Linear regression coefficient (slope), R^2 and p-values of ammonium, nitrite, nitrate and phosphate concentration (µg g_{DW}^{-1} L^{-1}) versus time (minutes) of uptake for seawater and different RAS-water treatments (% RAS-water). Low R^2-values and p-values > 0.05 (bold) indicates no interaction between nutrient concentration and time.

Nutrient	Treatment	Slope	R^2	p
Ammonium	Seawater	-0.1	0.56	**0.73**
	25 %	-7.0	0.36	< 0.001
	50 %	-13.0	0.29	0.001
	75 %	-16.9	0.32	0.001
	100 %	-28.4	0.70	< 0.001
Nitrite	Seawater	0.1	0.44	< 0.05
	25 %	-0.2	0.13	**0.22**
	50 %	-0.2	0.01	**0.51**
	75 %	-0.2	0.09	**0.59**
	100 %	-0.3	0.58	< 0.001
Nitrate	Seawater	-1.6	0.48	**0.38**
	25 %	-1.7	0.11	**0.39**
	50 %	-1.5	0.00	**0.37**
	75 %	1.9	0.07	**0.23**
	100 %	-11.3	0.56	**0.15**
Phosphate	Seawater	-0.1	0.49	< 0.05
	25 %	-0.4	0.12	< 0.05
	50 %	-1.9	0.00	**0.21**
	75 %	-3.3	0.10	**0.87**
	100 %	-3.1	0.52	< 0.001

A. Phosphate (PO_4^{3-})

Time did only have a significant effect on the phosphate concentration for treatment 100 %, 25 % and seawater ($p < 0.05$). Here, the phosphate concentrations decreased with time, indicating an uptake. Linear regression showed a significant ($p < 0.001$) relationship between nitrite concentration in the water and treatment, expect seawater ($p > 0.05$), as well as all treatments being significant ($p < 0.001$) different in phosphate concentration compared to each other (One-way ANOVA, Post Hoc Tukey's test).

3.1.2 Uptake rates

Uptake rates were calculated from start to endpoint (0 to 480 minutes) for every nutrient, with the formula given in Equation 2.3. Ammonium is the only nutrient from Table 3.2 and Figure 3.1 that is suggesting a significant decrease in concentration in the water over time for every RAS-gradient treatment. This resulted in only ammonium getting an increased uptake rate with increased nutrient concentration. Uptake rates of ammonium (V) normalized to dry weight ranged between 25 % and 100 % RAS-water from 95.4 ± 1.7 to 387.0 ± 18.1 µg NH_4^+ g_{DW}^{-1} hour $^{-1}$. This is equivalent to 6.8 ± 0.1 to 27.6 ± 1.3 µM NH_4 g_{DW}^{-1} $hour^{-1}$. Ammonium uptake revealed a strong linear relationship with increased ammonium concentration ($R^2 = 0.94$, $p < 0.001$). The uptake rate for ammonium over the course of the experiment is visually presented in Figure 3.2 and the uptake rate across treatments per time interval can be investigated in Table B.1 (Appendix B).

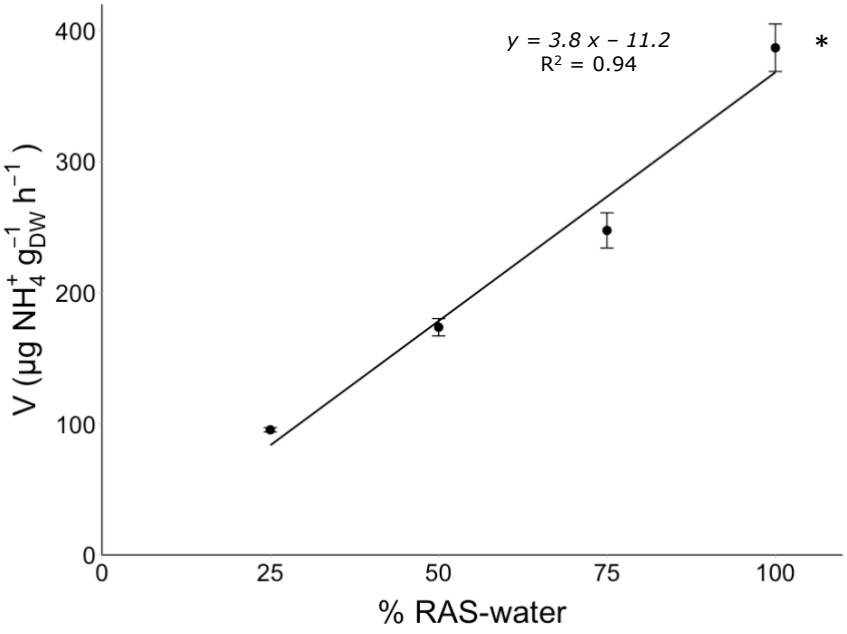

Figure 3.2: Uptake rates of NH_4^+ (V, µg g_{DW}^{-1} h $^{-1}$) for *Ulva* sp. as a function of initial ambient RAS-water concentrations. 100 % RAS-water equals 2213.8 ± 6.7 µg NH_4^+ L^{-1} ~158.1 ± 0.5 µM NH_4^+. Presented as mean ± SE (n = 4). Line represent linear regression through the data, expressed as *y = 3.8 x – 11.2*, where x is % RAS-water. * implies that every measured concentration of RAS-water being significantly different from each other.

For the nutrients where time did not have a significant effect on the nutrient concentration in the water, *Ulva* sp. was suggested to have low uptake rate throughout the total duration of the experiment. This applied for nitrite, nitrate and phosphate. However, the RAS-gradient was found to have a significant effect on the nitrate uptake rate (linear regression), even though one-way ANOVA suggested no significant difference in nitrate uptake rate between % RAS-water treatments. For nitrate, negative uptake rates were found for every treatment, ranging for treatment 25 % to 100 % from -45.7 ± 27.9 to -308.0 ± 73.9 µg g_{DW}^{-1} h^{-1}. This indicates higher nitrate substrate concentration at the end of the experiment compared to the initial concentration. Uptake rate values (V, µg g_{DW}^{-1} L^{-1}) are given in Table 3.3 and linear regression coefficient (slope), R^2 values and p-values for ammonium, nitrite, nitrate, and phosphate uptake versus RAS water gradient is given in Table 3.4. Visual presentations of nitrite, nitrate and phosphate uptake rates are given in Figure B.1 (Appendix B).

Table 3.3: Uptake rate (V, µg g_{DW}^{-1} h^{-1}) of ammonium, nitrite, nitrate and phosphate for *Ulva* sp. at different RAS-water concentrations given as mean ± SE (n = 4) over the course of the experiement (0-480 minutes). Negative values suggests higher water concentrations at end compared to start. Different letters within each nutrient suggests significant difference between RAS concentrations.

	V (µg g_{DW}^{-1} h^{-1})			
Nutrient	25 %	50 %	75 %	100 %
NH_4^+ (0-480 min)	95.4 ± 1.7^a	174.0 ± 6.6^b	248.0 ± 13.4^c	387.0 ± 18.1^d
NO_2^- (0-480 min)	2.9 ± 0.7^a	3.9 ± 1.1^a	5.1 ± 0.9^a	4.9 ± 1.9^a
NO_3^- (0-480 min)	-45.7 ± 27.9^a	-47.9 ± 60.0^a	-170.0 ± 116.0^a	-308.0 ± 73.9^a
PO_4^{3-} (0-480 min)	8.3 ± 1.4^{a,b}	-1.9 ± 8.3^a	47.4 ± 5.2^b	8.2 ± 16.8^{a,b}

Table 3.4: Linear regression coefficient (slope), R^2 values and p-values of ammonium, nitrite, nitrate and phosphate uptake (V, µg g_{DW}^{-1} L^{-1}) versus RAS-water gradient.

Nutrient	Time period (min)	Slope	R^2	p
Ammonium	0-480	3.8	0.94	< 0.001
Nitrite	0-480	0.0	0.06	**0.19**
Nitrate	0-480	-3.6	0.31	< 0.05
Phosphate	0-480	0.2	0.04	**0.42**

3.2 Density gradient (Experiment II)

3.2.1 Substrate concentration

Nutrient concentration in the water for the different treatments (0.25, 0.5, 1.3, 2 and 4 g_{WW} per 250 mL ~ 1, 2, 8 and 16 g_{ww} per 1 L) over a ten-hour time period (600 minutes) is shown in Figure 3.3. The percentage decrease in nutrients in the water from start (0 minutes) to end (600 minutes) can be investigated in Table A.3 (Appendix A).

Ammonium, nitrite, nitrate and phosphate concentrations (µg L^{-1}) in relation to time for every density treatment can be investigated in Figure A.1 (Appendix A).

A. Ammonium (NH_4)

Linear regression showed a significant relationship ($p < 0.05$) between ammonium concentration (µg g_{DW}^{-1} L^{-1}) in the water and time for all treatments. The treatment with lowest density (0.25 g_{WW}) decreased most in ammonium concentration over time, with a slope of -5.9 (Table 3.5). Since the water concentrations were calculated per g_{DW}, the slopes tended to have a less steep slope with increased density as the initial concentration changed related to initial biomass. Treatments 1.3-2 g_{WW}, 1.3-4 g_{WW} and 2-4 g_{WW} did not significantly differ in ammonium concentration compared to each other (One-way ANOVA, Post Hoc Tukey's test).

B. Nitrite (NO_2^-)

For nitrite, no significant interaction effect between time and treatment was found ($p > 0.05$). Within the time period 0 to 20 minutes, the same dip as observed in Experiment I applied. A decrease was then observed, and a sustained water concentration was detected throughout the rest of the experimental period.

C. Nitrate (NO_3^-)

Time did not have any significant effect on the nitrate concentration throughout the ten-hour experiment. Nitrate concentration was from Figure 3.1 visually shown to decrease with time in the first 80 minutes for all treatments, before the concentration in the water increased again. Hence, it was decided to run a separate linear regression model on the first 80 minutes (Table 3.5), and here a linear regression model showed a significant ($p < 0.001$) relationship between nitrate concentration in the water and time for all treatments, indicating an uptake of nitrate.

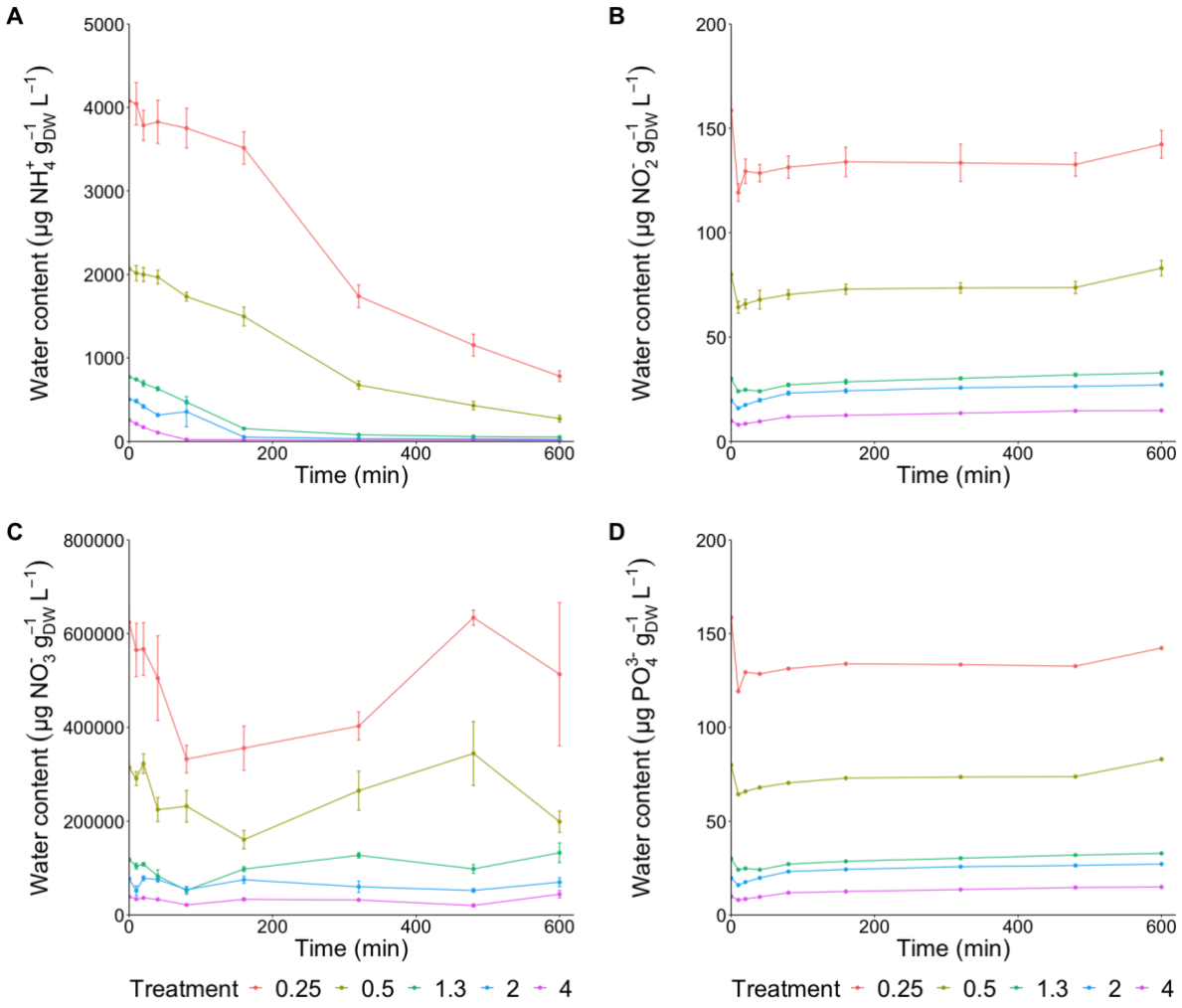

Figure 3.3: Water content concentrations (µg g_{DW}^{-1} L^{-1}) of A) Ammonium, B) Nitrate, C) Nitrite and D) Phosphate for each treatment (0.25, 0.5, 1.3, 2 and 4 g_{WW} per 250 mL volume) at different timepoints expressed at mean± SE (n = 4). Notice the different values on the y-axis.

D. Phosphate (PO_4^{3-})

Time did not have any significant effect on the phosphate concentration, indicating no uptake. All the treatments showed a significant effect on the phosphate concentration ($p < 0.001$), as well as all treatments being significant different from each other.

Table 3.5: Linear regression coefficient (slope), R^2 and p-values of ammonium, nitrite, nitrate and phosphate concentration (µg g_{DW}^{-1} L^{-1}) versus time (minutes) of uptake for different density treatments (g_{WW}). Low R^2-values and p-values > 0.05 (bold) indicates no interaction between nutrient concentration and time.

Nutrient	Treatment	Slope	R^2	p
Ammonium	0.25 g	-5.9	0.81	< 0.001
	0.5 g	-3.2	0.16	< 0.001
	1.3 g	-1.2	0.20	< 0.001
	2 g	-0.8	0.24	< 0.001
	4 g	-0.3	0.29	< 0.001
Nitrite	0.25 g	0.0	0.77	**0.25**
	0.5 g	0.0	0.03	**0.24**
	1.3 g	0.0	0.07	**0.54**
	2 g	0.0	0.11	**0.32**
	4 g	0.0	0.20	**0.69**
Nitrate	0.25 g	17.9	0.64	**0.78**
	0.5 g	-37.6	0.02	**0.56**
	1.3 g	46.5	0.04	**0.76**
	2 g	-12.8	0.09	**0.74**
	4 g	0.5	0.16	**0.85**
Nitrate (0-80 min)	0.25 g	-3505.3	0.73	< 0.001
	0.5 g	-1158.5	0.04	< 0.001
	1.3 g	-813.1	0.07	< 0.001
	2 g	-168.5	0.12	< 0.001
	4 g	-201.1	0.19	< 0.001
Phosphorus	0.25 g	-0.9	0.78	**0.36**
	0.5 g	0.7	0.02	**0.26**
	1.3 g	0.1	0.05	**0.48**
	2 g	-1.4	0.12	**0.73**
	4 g	-1.2	0.20	**0.84**

3.2.2 Uptake rates

Uptake rates of ammonium (V) normalized to dry weight ranged throughout the experiment between density treatments 0.25 g and 4 g from 82.4 ± 2.7 to 6.0 ± 0.0 µg NH_4^+ g_{DW}^{-1} hour $^{-1}$, equivalent to 5.9 ± 0.2 to 0.4 ± 0.0 µM NH_4^+ g_{DW}^{-1} hour $^{-1}$ (Table 3.6). This implied a decrease in uptake rate with an increase in density. Thus, ammonium uptake revealed a strong exponential decay with increased biomass density (R^2 = 0.87, p < 0.001), giving the exponential regression model presented in Equation 3.1, where y is the ammonium uptake rate (V, µg g_{DW}^{-1} h $^{-1}$) and x is the density (g_{WW}) per 250 mL. The uptake rate for ammonium over the course of the Experiment II is visually presented in Figure 3.4 A and uptake rate across treatments for each time interval can be investigated in Table B.3 (Appendix B).

$$y = 61.6 * 0.5^x \quad (3.1)$$

Uptake rates of nitrate (V) normalized to dry weight ranged between timepoint 0 and 80 minutes between density treatments 0.25 g and 4 g from 54 748 ± 7 366 to 1 932 ± 701 µg NO_3^- g_{DW}^{-1} hour $^{-1}$, equivalent to 3911 ± 526 to 138 ± 50 µM NO_3^- g_{DW}^{-1} hour $^{-1}$ (Table

3.6). Uptake rate of nitrate across treatments for each time interval can be investigated in Table B.4 (Appendix B). The same trend as ammonium in uptake rate in relation to density applied also for nitrate in the first 80 minutes, with a exponential decrease in uptake with increased biomass density (R^2 = 0.86, p < 0.001). The regression model for nitrate between timepoint 0 and 80 minutes is presented in Equation 3.2 and uptake rate for the same time interval is shown in Figure 3.4 B.

$$y = 28853.9 * 0.5^{x} \quad (3.2)$$

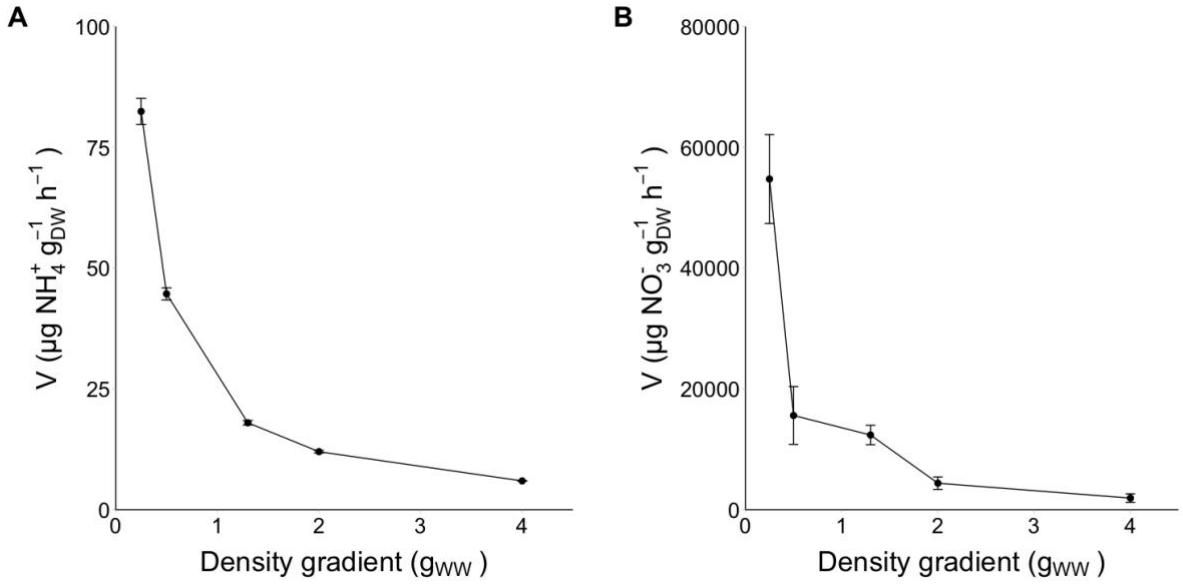

Figure 3.4: Uptake rates of A) NH_4^+ (V, µg g_{DW}^{-1} h^{-1}) in the time interval 0-600 minutes and B) NO_3^- (V, µg g_{DW}^{-1} h^{-1}) in the time interval 0-80 minutes for *Ulva* sp. as a function of algae densities (g_{WW} per 250 mL) in RAS-water with initial concentrations of 229.0 ±8.5 µg NH_4^+ L^{-1} and 35 013.1 ± 1476.3 µg NO_3^- L^{-1}. Notice different values on y-axis.

Table 3.6: Uptake rates (V, µg g_{DW}^{-1} h^{-1}) of ammonium, nitrite, nitrate and phosphate for *Ulva* sp.at different biomass densities given as mean ± SE (n = 4). Negative values suggests higher water concentrations at end compared to start. Different letters within each nutrient suggests significant difference between RAS concentrations.

	V (µg g_{DW}^{-1} h^{-1})				
Treatments	0.25 g	0.5 g	1.3 g	2 g	4 g
NH_4^+ (0-600 min)	82.4 ± 2.7^{a}	44.7 ± 1.2^{b}	18.0 ± 0.5^{c}	12.0 ± 0.3^{d}	6.0 ± 0.0^{e}
NO_2^- (0-600 min)	0.4 ± 0.1^{a}	-0.1 ± 0.1^{a}	-0.1 ± 0.0^{a}	-0.2 ± 0.0^{a}	-0.13 ± 0.0^{a}
NO_3^- (0-80 min)	54 748 ± 7 366^{a}	15 609 ± 4 786^{b}	12 367 ± 1 602^{c}	4 396 ± 1 030^{c,d}	1 932 ± 701^{d}
PO_4^{3-} (0-600 min)	-28.6 ± 19.7^{a}	-17.1 ± 11.3^{a,b}	1.4 ± 11.3^{a,b}	25.0 ± 9.7^{b}	3.7 ± 3.3^{a,b}

Table 3.7: R^2 and p-values of ammonium, nitrite, nitrate and phosphate uptake (V, µg g_{DW}^{-1} L^{-1}) versus density gradient.

Nutrient	Model	Time period (min)	R^2	p
Ammonium	Exponential	0-600	0.87	< 0.001
Nitrite	Exponential	0-600	0.59	< 0.05
Nitrate	Exponential	0-80	0.86	< 0.001
Phosphate	Exponential	0-600	0.03	**0.29**

Density was found to significantly influence the uptake rate of nitrite as well, where an exponential regression model was discovered to suit the data best (R^2 = 0.59). However, uptake values were relatively low for nitrite, ranging between -0.2 ± 0.0 µg NO_2^- g_{DW}^{-1} $hour^{-1}$ for 2 g_{WW} and 0.4 ± 0.1 µg NO_2^- g_{DW}^{-1} $hour^{-1}$ for 0.25 g_{WW}, resulting in neither density being significantly different in uptake from each other ($p > 0.05$, One-way ANOVA, Table 3.6). R^2 and p-values gained from the exponential regression model for all nutrients are presented in Table 3.7.

Visual presentation of nitrate and phosphate uptake across densities can be investigated in Figure B.2 (Appendix B).

3.3 Tissue content

Mean tissue content (% C, N and P of DW) in *Ulva* sp. samples from Experiment I and II is presented in Figure 3.5 and values are given in Table C.1 (Appendix C). Initial carbon (C), nitrogen (N) and phosphorus (P) content prior incubation were measured from bulk samples (n = 3) of *Ulva* sp. kept in deep sea water tanks, and estimated to be 25 ± 3, 3 ± 0.4 and 5 ± 0.6 % C, N and P of DW for experiment I and 25 ± 3, 2 ± 0.2 and 2 ± 0.2 % C, N and P of DW Experiment II, respectively. For both experiments, tissue C, N and P did not differ significantly ($p > 0.05$) between the different RAS-water concentration and different densities, or between the initial content in the tissue of *Ulva* sp. and the treatments (One-way ANOVA, Post Hoc Tukey's test).

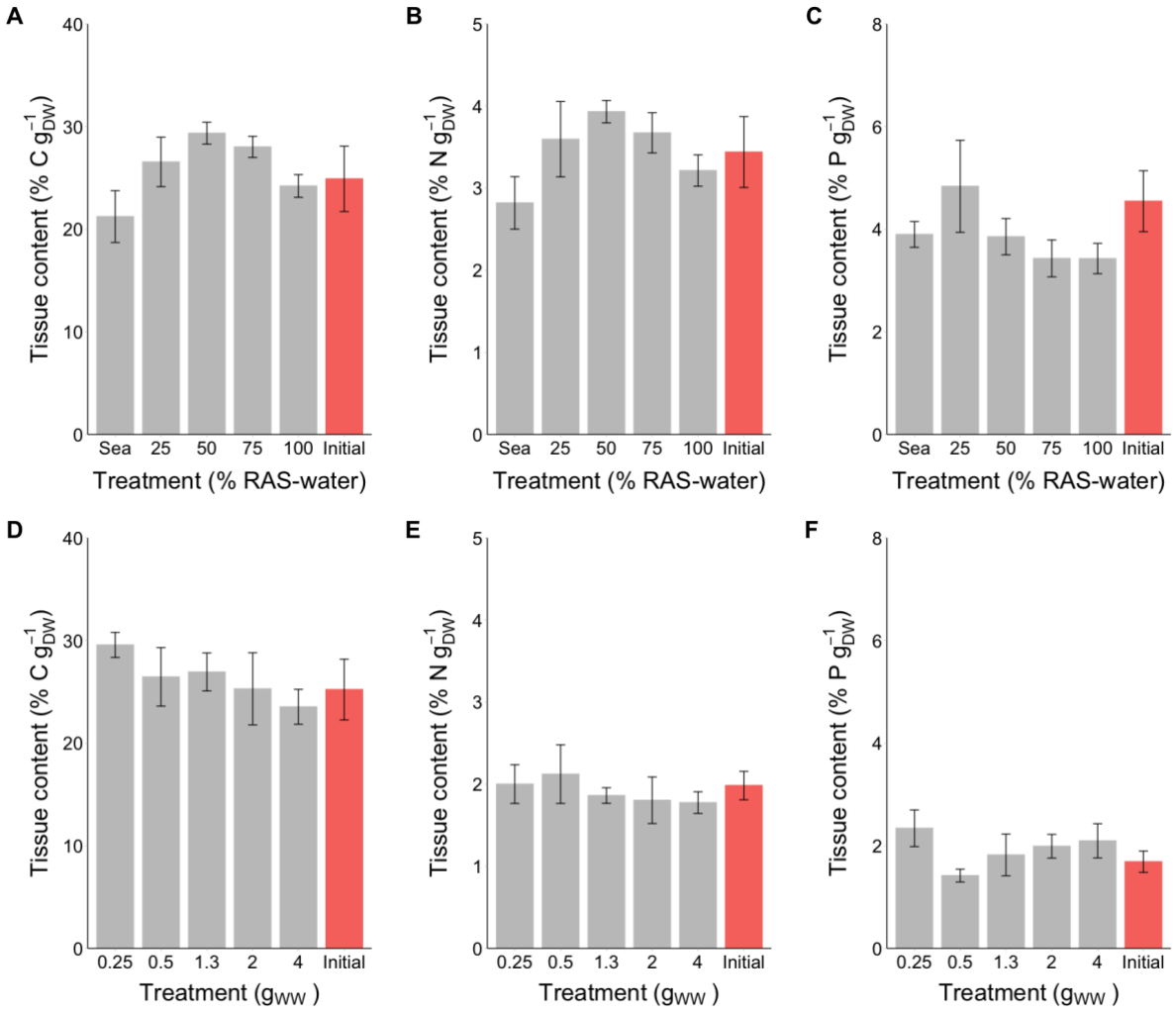

Figure 3.5: Tissue content as % per g_{DW} of A) Carbon (C, % RAS-water treatment), B) Nitrogen (N, % RAS-water treatment), C) Phosphorus (P, % RAS-water treatment), D) Carbon (C, Biomass-treatment), E) Nitrogen (N, Biomass-treatment) and F) Phosphorus (P, Biomass-treatment) compared to initial content (control) expressed as mean± SE (n = 4 for treatments, n = 3 for initial controls). Notice different values on y-axis.

Neither the C:N ratio or N:P ratio in Experiment I and II did significantly differ between different treatments or between treatments and initial ratios (One-way ANOVA, $p > 0.05$). Initial C:N ratio was estimated to be 7 (± 0.2):1 in Experiment I and 13 (± 0.4):1 in experiment II. Initial N:P ratio was found to be 0.8 (± 0.2):1 and 1.2 (± 0.25):1 for Experiment I and II, respectively. C:N ratio and N:P ratio for experiment I and II is presented in Figure 3.6.

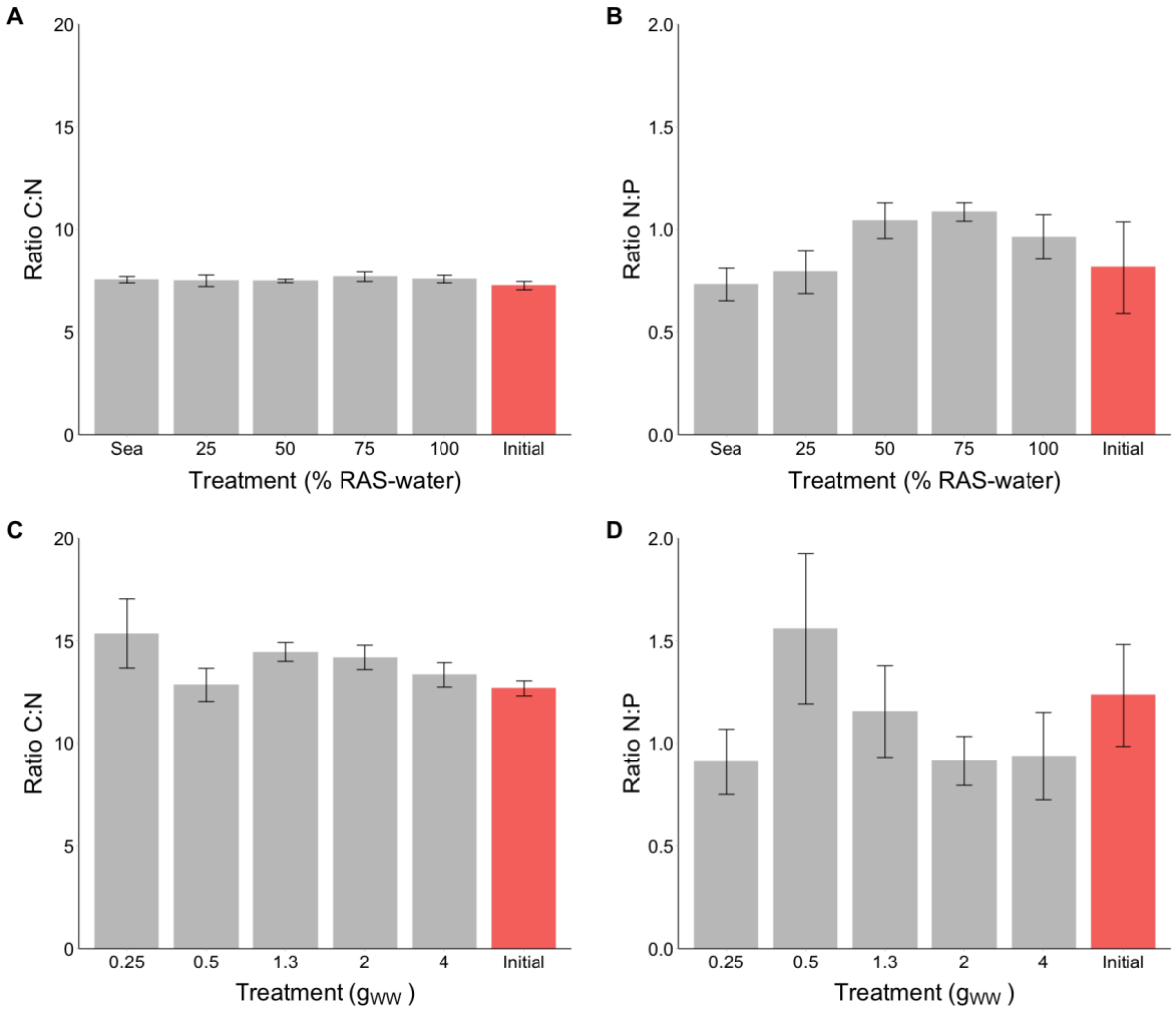

Figure 3.6: C:N ratios in Ulva sp. exposed to A) different RAS-water gradients and C) density gradients. N:P ratios in Ulva sp. exposed to C) different RAS-water gradients and D) density gradients. Values expressed as mean± SE (n = 4 for treatments, n = 3 for initial controls). Notice difference in values on y-axis between C:N and N:P ratios.

4 Discussion

In this study, the bioremediation potential and initial uptake rate of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphorus (PO_4^{3-}) from wastewater of Recirculating Aquaculture Systems (RAS) were investigated in the opportunistic green algae *Ulva* sp. NH_4^+ and NO_3^- serves as the main components in the RAS medium after treatment in the biofilter, and most attention has and will be given to these two nitrogen sources. The optimal macroalgal species to be used for bioremediation of nutrients in RAS will be one having tolerance for lower salinities compared to natural seawater, as well as high uptake rate of nitrogen, and especially in the form of nitrate. This is the inorganic nitrogen compound that exist in highest concentration in the wastewater. Thus, by being able to remove this nutrient, it will increase the cleaning potential remarkable.

In the experiments conducted in the current study, *Ulva* sp. received much higher nitrogen concentrations (Table A.1, Appendix A) and lower salinities compared to natural seawater conditions. Nutrient concentration in relation to time revealed the same trend for all RAS-gradients treatments (Figure 3.1), suggesting that the macroalgae had the same response to both higher (100 % RAS-water) and lower nutrient concentrations (25 % RAS-water). Also, the salinity of ~ 15 ppt did not appear to be a challenge regarding nitrogen uptake as most of the ammonium got taken up by *Ulva* sp. in both experiments. The ability to grow in a wide range of salinities (10-48 ppt; Rybak, 2019) can be seen as one of the core qualities of *Ulva* sp. compared to other macroalgae. In such case, it is a very suitable species to bioremediate nutrients from RAS wastewater, as this water normally has lower salinity ranges compared to natural seawater due to farming Atlantic salmon (*Salmo salar*) smolt and post smolt.

4.1 Uptake rates of NH_4^+ and NO_3^- in *Ulva* sp. cultured in high nitrogen medium

The affinity for ammonium in *Ulva* sp. was in Experiment I demonstrated to be higher than for nitrate, which is supported in several other uptake experiments conducted for this macroalgae (e.g., Naldi & Wheeler, 2002; Ale et al., 2011; Luo et al., 2012; Martinez et al., 2012; Shpigel et al., 2019). Above 96 % of the ammonium from the RAS wastewater was removed at concentrations up to ~ 160 µM, which is equivalent to 100 % RAS wastewater in Experiment I (Table A.2, Appendix A). For nitrate, no significant decrease was found during the experimental period, indicating that ammonium was the only nitrogen source that *Ulva* sp. took up. The higher affinity for ammonium is possible related to less energy required to take up and assimilate the nutrient, considering that nitrate actively must be reduced to ammonium by nitrate reductase before assimilation into macro molecules, such as amino acids (Ale et al., 2011; Luo et al., 2012). However, a possible trend in uptake of nitrate could have been seen if the same experiment was performed over a longer period. In an experiment performed with *Saccharina latissima* offered both ammonium and nitrate (Etter et al., unpublished data), the algae were found to take up ammonium until the concentration became low (~ 3 µM), which is below the concentration at 480 minutes in this current study. After, an uptake of nitrate was discovered in *Saccharina latissima*.

This study demonstrated unsaturable ammonium uptake kinetics during the experimental period of 8 hours with substrate concentrations up to 160 µM (100 % RAS wastewater).

Ammonium uptake rate was therefore in Experiment I found to be linearly related to substrate concentration ($R^2 = 0.94$, $p < 0.001$), which can be explained by the passive diffusion of the nutrient across the cell membrane (Taylor et al., 1998; Philips & Hurd, 2004; Hurd et al., 2014). These findings are supporting hypothesis 1 and 2 (see chapter 1.4). However, saturable ammonium uptake kinetics in *Ulva lactuca*, *U. prolifera* and *U. linza* have in a few studies been reported at ammonium concentrations of 72-200 µM (Pedersen, 1994; Luo et al., 2012). This indicates that saturation can be met for higher ammonium concentrations than tested for in this study or that uptake is very species or geographical specific, and that variations perhaps occur depending on factors like age and nutritional history.

Experiment II, investigating biomass gradients, was mainly conducted to establish the most effective density to bioremediate nutrients with *Ulva* sp., as there seemed to be a big variation in densities used in previous studies (e.g., Cohen & Neori, 1991; Pedersen, 1994; Pedersen & Borum, 1997; Mártinez et al., 2012).

Nitrogen uptake in *Ulva* sp. appeared to be density dependent, where ammonium and nitrate uptake rate per unit biomass (g_{DW}) were higher in the lower stocking density (0.25 g_{WW}), which can be explained by more nutrients being available per gram of algae in the lower densities, compared to the higher ones. However, increasing density of *Ulva* sp. led to a more rapid decrease in the ammonium concentration (µg NH_4 L^{-1}) with time (Figure A.1 A, Appendix A), which is supported by Vandermulen & Gordin (1990). For the density of 4 g_{WW} per 250 mL, almost all the ammonium was depleted from the medium after 80 minutes with no further uptake (Table B.3, Appendix B), while the lower densities used more time to take up the ammonium and depleting it from the medium. This indicates a steadier uptake throughout the experiment for densities 0.25 g_{WW}, 0.5 g_{WW}, 1.3 g_{WW} and 2 g_{WW}, while 4 g_{WW} took up all the nutrients more rapidly. Hence, there were not enough ammonium in the water for the 4 g_{WW} density to have a continuous uptake throughout the experimental period. This may suggest that the lower densities tested for in this study will be more effective in a setup like the one performed in this experiment, as approximately the same amount of nutrients can be removed with less biomass. However, in a more realistic scenario when using *Ulva* sp. as an organism to bioremediate nutrients from RAS wastewater, the macroalgae will probably be exposed to nutrients continuously (Cohen & Neori, 1991), and higher densities will hence not be able to deplete all the nutrients from the RAS medium.

In both experiments performed in the current study, *Ulva* sp. was exposed to an interaction of the two major nitrogen sources, ammonium and nitrate. In Experiment I, no significant uptake with increased nitrate availability was found, but nitrate uptake was registered in Experiment II between 0 and 80 minutes.

The two uptake experiments conducted in this study varied in initial nutrient concentrations (Table 3.1), where the largest difference was found in the nitrate:ammonium ratio. Wastewater from RAS used in Experiment I had 12 times higher nitrate concentrations compared to ammonium, while the nitrate:ammonium ratio was found to be 152 in Experiment II. These values can contribute to explain the rapid decrease in nitrate substrate concentration for the first 80 minutes in Experiment II, suggesting that ammonium was not able to inhibit nitrate uptake when nitrate was found in such large concentration compared to ammonium. This reflection is supported by Iwasaki (1967), which noted that the apparent preference of one nitrogen source over another in the

Conchocelis life stage of the red algae *Porphyra* sp. was dependent on the concentration used in the medium and that ammonium was found to only inhibit nitrate when it reached a certain concentration compared to nitrate. Fan et al. (2013) also reported that the NO_3^-:NH_4^+ ratio clearly influenced the nitrate and ammonium uptake rates in *U. prolifera*. However, Fan et al. (2013) found that this macroalgae preferred ammonium as nitrogen source over nitrate when the nitrate:ammonium ratio was less than 2.2. This cannot be confirmed in this study, as the affinity for ammonium over nitrate was found at a ratio of 12. These findings may indicate that the utilization of ammonia and nitrate by macroalgae is species-specific within the *Ulva* sp. genus and hence an important study area.

The increase in nitrate content after the dip at 80 minutes in Experiment II may also imply that the storage ability can have reached a saturation point. Lartigue & Sherman (2005) described that as the internal pools fill during nitrate uptake, activity of nitrate reductase (NR) increases, and a decrease in uptake rate could be caused by a feedback inhibition of the uptake system. However, few studies have revealed a release of nutrients when the algae is reaching a saturation point, and a stable uptake is found to be more common when saturated (Figure 1.3 B) (Pedersen & Borum, 1997; Luo et al., 2012; Martinez et al., 2012; Li et al., 2016). These results may therefore indicate that another process have happened, and stress from high nutrient supply could be a reasonable explanation, causing nitrogen to leak out of the cells. However, nitrate concentrations in wastewater from RAS (~ 1950-2500 µM) is above the range used in many previous studies (e.g., Ale et al., 2011; Luo et al., 2012; Shpigel et al., 2019) and a comparison of algae responses to such high nitrogen concentrations is hence hard to perform.

4.2 Initial tissue content vs. tissue content after high nitrogen exposure in *Ulva* sp.

This study revealed no significant difference in tissue content after treatment compared to the initial tissue content, even though the intracellular nitrogen content has been found to be related to the ambient nitrogen concentrations in *Ulva* sp. (Cohen & Neori, 1991; Nielsen et al., 2012). This applied for both experiments performed and suggests that *Ulva* sp. did not store reserves of intracellular nitrogen after exposure to RAS wastewater with high nitrogen concentrations. The storage capacity of opportunistic macroalgal species with high surface area:volume (SA:V) ratio, as *Ulva* sp., has the potential to be seen in relation with these results. High SA:V ratio species tend to have high maximum uptake rates (V_{max}), but on the cost of storage capacity (Littler & Littler, 1980).

Despite no difference compared to initial content, carbon:nitrogen (C:N) ratio and tissue nitrogen (% DW) values varied among the two experiments conducted in this study. This can be seen in relation to the different seasons these macroalgae were harvested (April for Experiment I and September for Experiment II). In spring, before phytoplankton spring bloom, more nitrogen is available in the water column compared to summer season (Lyngby & Mortensen, 1994; Pedersen & Borum, 1997), resulting in a higher initial C:N ratio and tissue nitrogen for the algae used in Experiment II. Initial C:N ratio was estimated to be ~ 7:1 and ~ 12:1 in Experiment I and II, respectively. Both these values are within the ratios reported for macroalgae in its natural environment (5:1-50:1) (Hanisak, 1983). However, Fujita (1985) described that C:N ratio content in *U. lactuca* grown at high nitrogen treatments was 8:1, and higher C:N ratios than 10:1-18:1 have been described to indicate nitrogen limitations (Hanisak, 1983; Björnsäter & Wheeler, 1990). Based on the

estimated C:N ratio, *Ulva* sp. used in Experiment I was therefore suggested to be nitrogen saturated, but more towards a nitrogen limited state in Experiment II. In addition, tissue nitrogen in *Ulva* sp. was found to vary between 2.8-3.9 in Experiment I and 1.8-2.1 in Experiment II. Nitrogen content in Experiment I is hence suggested to be above the value that is thought to be the value for saturated growth for various *Ulva* species (2-3 % of DW) (Fujita et al., 1989; Björnsäter & Wheeler, 1990; Lavery & McComb, 1991; Campbell, 2001). The initial saturated conditions for *Ulva* sp. used in Experiment I could make *Ulva* sp. not using energy on storing nutrients but rather used to growth (Campbell, 2001), and a significant increase in tissue content compared to initial content was therefore not observed.

Visual inspection showed that the algae harvested in September were less fresh looking and smaller in size compared to the ones harvested in the spring. It is common for *Ulva* species to disintegrate during summer after sporulation (Fujita, 1985; Bruhn et al., 2011), but nutrient uptake was found according to removal of nutrients in the substrate and does therefore not serve as a reasonable explanation. However, survival during periods of nutrient limitation is shown to be a balance between storage capacity and growth rate (Fujita, 1985). Since *Ulva* is a species featuring high growth rates, a possibly explanation to the results found in this study could therefore be explained by the low capacity for buffering nutrient concentrations fluctuations in the surrounding environment (Campbell, 2001). However, Vlottes (unpublished data), reported an increase in tissue nitrogen *Ulva* sp. within six days after exposure to high nitrogen concentration and was also found for the brown kelp *Saccharina latissima* (Forbord et al., 2021). Hence, tissue nitrogen content could be a bad indicator of uptake kinetics in this study, as tissue nitrogen provide a more long-term index of the algae nutritional state (Fong et al., 1994).

4.3 Bioremediation potential of *Ulva* sp. and implications for IMTA

Using RAS for smolt production of Atlantic salmon has increased in Norway the past years. There is also a growing trend towards farming of salmon in RAS after smoltification (post smolts) to improve their survival and robustness after transfer to sea cages (Dalsgaard et al., 2013). *Ulva* sp. demonstrated in the current study to successfully extract nitrogen compounds from the RAS wastewater and serve therefore as a possible organism to be incorporated in RAS and opens for opportunities to lessen the amount of nitrogen in the discharged wastewater. Hence, it can contribute to a more sustainable aquaculture industry, both environmentally and economically. The environmental sustainability can be improved by reducing nutrient wastage and thus reduce the negative impact on the environment, while the economically sustainability can be enhanced by producing additional biomass without the need for supplementary feeding.

To improve the environmental sustainability of RAS, a reduction in the amount of phosphorus in the water effluent (Martins et al., 2010) together with the nitrogen removal would be beneficial. Regarding phosphorus uptake in this study, a minor uptake of phosphate from the RAS-water was found for treatment 100 % RAS-water in Experiment I but was not observed in Experiment II. Thus, this study was not able to demonstrate any precise phosphate uptake, which got dominated by the larger amounts of nitrogen compounds. However, ammonium and nitrate do serve as a health threat to fish in RAS, while phosphorus is not considered to be directly toxic (Carpenter et al., 1998). In case of

removal of toxic compounds from RAS water, an uptake of phosphorus in *Ulva* sp. from the water is not essential, and a circular and sustainable production can still be obtained.

While the present study has determined that *Ulva* sp. are able to bioremediate nitrogen sources from RAS wastewater, future studies on incorporation strategies in RAS and potential market value and growth potential in RAS medium is recommended to fully determine the possible implications for IMTA with *Ulva* sp.

4.4 Challenges, limitations and future work

Ulva sp. has a diplohaplontic sexual cycle, with isomorphic life stages (Alström-Rapaport, 2010). This results in similar morphology between the gametophyte and sporophyte which cannot be distinguished with visual inspection. Hence, it is unknown which part of the sexual cycle that took place in this study and if bioremediation potential would vary with different life stages. To integrate production of *Ulva* sp. in RAS, it is recommended investigation on potentials for these species to be cultivated with fully control on this aspect.

As the bioremediation potential relies on the metabolic activity of *Ulva* sp., it is important that the culturing environment provides optimal resources for the algae to grow and thrive (Neveux et al., 2017). It would be more optimal to run Experiment I (RAS-water gradient) with a water that contained more nitrate and less ammonium. In such case, a more realistic picture of the possibility of *Ulva* sp. to clean wastewater from RAS would be presented. Substrate concentrations in the water used in Experiment II are most representative for values in wastewater from Norwegian salmon RAS-wastewater and the nitrogen uptake was found to vary with the quality of the biofilter. Uptake of nitrate was never proven in Experiment I, but a possible trend could maybe be seen with water that contained a higher nitrate:ammonium ratio. Then a potential saturated nitrate uptake with increased concentration may have applied and could easier been seen in comparison with other studies.

5 Conclusion

The present study has contributed to a better understanding of the application of *Ulva* sp. as a bioremediating component in wastewater from Recirculating Aquaculture Systems (RAS) farming Atlantic Salmon (*Salmo salar*). *Ulva* sp. was found to be a suitable candidate in bioremediating nitrogen compounds, and the results do therefore recognize the incorporation of this macroalgae in RAS as an opportunity to lessen the amount of nitrogen in the discharged wastewater.

Ulva sp. expressed high initial nitrogen uptake and had a significant linear increase in ammonium uptake with increasing substrate concentrations, validating Hypothesis 2: «*The ammonium (NH_4^+) uptake rate will increase linearly with increased exposure concentration».* Preferred nitrogen source in *Ulva* sp. was strongly affected by the ratio of available nitrate and ammonium in the wastewaters from RAS. At low nitrate:ammonium ratios (12:1), ammonium was found the be the favored nitrogen source regarding uptake, and consequently inhibited the uptake of nitrate, confirming Hypothesis 1: «*Ulva sp. will favor ammonium (NH_4^+) as a nitrogen source over nitrate (NO_3^-)».* However, at a higher nitrate:ammonium ratio (152:1), an uptake in nitrate was discovered, indicating that this macroalgal species holds the potential to remove the nitrogen source, nitrate, which exists in highest concentrations in wastewater from RAS. This will serve as the most optimal scenario to use *Ulva* sp. as a cleaning solution.

Results revealed in the present study does consequently suggests that IMTA with *Ulva* sp. has the possibility to offer an opportunity in secondary production of useful biomass. Further research on the potential market value and growth rates in RAS medium of *Ulva* sp. is hence recommended.

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Appendix A: Substrate concentrations

Table A.1: Mean ± SE (n = 4) nutrient values (µg L^{-1} and µM) for null samples from Experiment I with RAS gradients and seawater (E1) and Experiment II with biomass gradients (E2).

Nutrient	Null Control	Mean value (µg L^{-1})	Mean value (µM)
Ammonium (NH_4^+)	100 % RAS E1	2213.8 ± 6.7	158.1 ± 0.5
	75 % RAS E1	1552.1 ± 37.1	110.9 ± 2.7
	50 % RAS E1	1071.8 ± 9.4	76.6 ± 0.7
	25 % RAS E1	559.9 ± 3.4	40.0 ± 0.2
	Seawater E1	8.1 ± 0.1	0.6 ± 0.0
	RAS E2	229.0 ± 8.5	16.4 ± 0.6
Nitrite (NO_2^-)	100 % RAS E1	313.4 ± 2.7	22.4 ± 0.2
	75 % RAS E1	238.5 ± 1.8	17.0 ± 0.1
	50 % RAS E1	154.9 ± 1.7	11.1 ± 0.1
	25 % RAS E1	78.1 ± 0.2	5.6 ± 0.0
	Seawater E1	1.2 ± 0.1	0.1 ± 0.0
	RAS E2	8.9 ± 0.3	0.6 ± 0.0
Nitrate (NO_3^-)	100 % RAS E1	27 239.2 ± 1052.1	1945.7 ± 75.2
	75 % RAS E1	18 999.0 ± 246.9	1357.1 ± 17.6
	50 % RAS E1	13 709.4 ± 164.5	979.2 ± 11.8
	25 % RAS E1	7321.7 ± 70.4	523.0 ± 5.0
	Seawater E1	158.0 ± 0.4	11.3 ± 0.0
	RAS E2	35 013.1 ± 1476.3	2500.9 ± 105.5
Phosphate (PO_4^{3-})	100 % RAS E1	1940.8 ± 129.7	62.7 ± 4.2
	75 % RAS E1	1654.8 ± 23.6	53.3 ± 0.8
	50 % RAS E1	1021.0 ± 35.8	33.0 ± 1.2
	25 % RAS E1	609.8 ± 16.3	19.7 ± 0.5
	Seawater E1	24.9 ± 0.2	0.8 ± 0.0
	RAS E2	1411.4 ± 20.3	45.6 ± 0.7

Table A.2: Percentage decrease of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}) in sea- and RAS-water from start (0 minutes) to end (480 minutes) expressed as mean ± SE (n = 4). Negative values indicates higher concentration at the end compared to the initial concentration.

Treatment (% RAS)	NH_4^+ (%)	NO_2^- (%)	NO_3^- (%)	PO_4^{3-} (%)
Seawater	-3.7 ± 15.5	-372.0 ± 113.0	59.1 ± 22.4	-11.7 ± 29.7
25 %	98.4 ± 0.2	21.2 ± 4.5	-2.4 ± 3.6	7.7 ± 2.1
50 %	96.6 ± 1.6	15.3 ± 4.8	-4.0 ± 1.4	-1.3 ± 3.5
75 %	96.4 ± 1.8	12.8 ± 2.5	-7.7 ± 1.9	17.3 ± 2.1
100 %	96.3 ± 2.0	9.3 ± 3.6	2.9 ± 3.8	-0.1 ± 9.9

Table A.3: Percentage decrease of ammonium (NH_4), nitrite (NO_2^-), nitrate (NO_3) and phosphate (PO_4^{3-}) for different density-treatments in water from start (0 minutes) to end (600 minutes) expressed as mean ± SE (n = 4). Negative values indicates higher concentration at the end compared to the initial concentration.

Treatment (g WW)	NH_4^+ (%)	NO_2^- (%)	NO_3^- (%)	NO_3^- (%) (0-80 min)	PO_4^{3-} (%)
0,25	80.8 ± 1.3	10.1 ± 3.8	21.0 ± 17.7	46.1 ± 6.0	-4.5 ± 3.7
0,5	87.0 ± 1.1	-4.0 ± 4.9	35.4 ± 10.5	26.9 ± 8.3	-5.0 ± 2.5
1,3	93.1 ± 1.4	-9.4 ± 4.4	-13.7 ± 10.7	56.2 ± 5.5	0.6 ± 10.4
2	95.3 ± 0.3	-38.4 ± 4.3	10.0 ± 10.7	29.3 ± 10.0	32.7 ± 13.3
4	94.2 ± 0.3	-50.9 ± 2.7	-16.1 ± 22.3	44.7 ± 5.9	47.6 ± 8.5

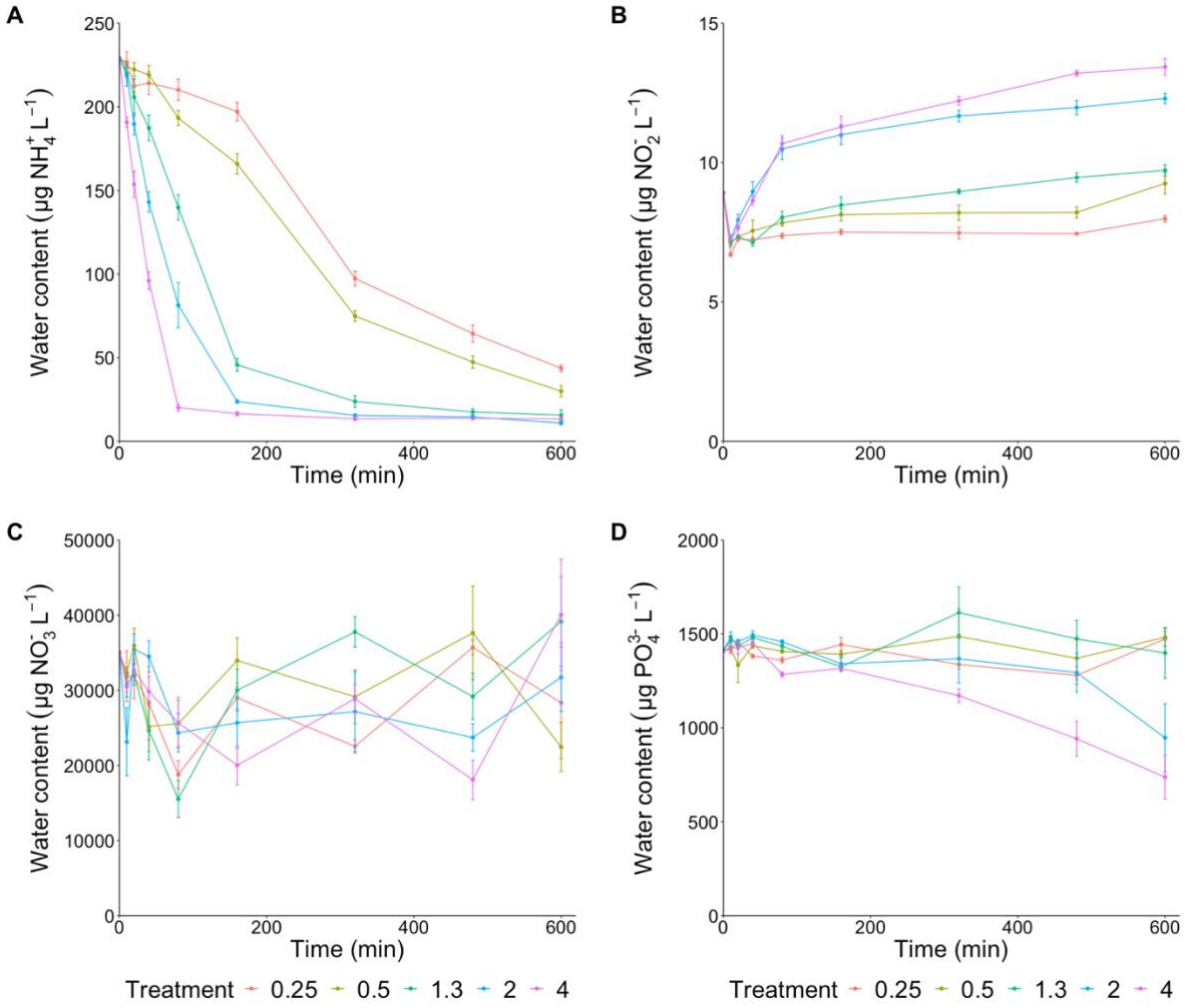


Figure A.1: Water content concentrations (µg L^{-1}) of A) Ammonium, B) Nitrite, C) Nitrate and D) Phosphate for each treatment (0.25 g_{WW}, 0.5 g_{WW}, 1.3 g_{WW}, 2 g_{WW} and 4 g_{WW} per 250 mL at different timepoints (minutes) expressed at mean± SE (n = 4). Notice the different values on y-axis.

Appendix B: Uptake rates

B.1 Uptake Experiment I: RAS-water gradient

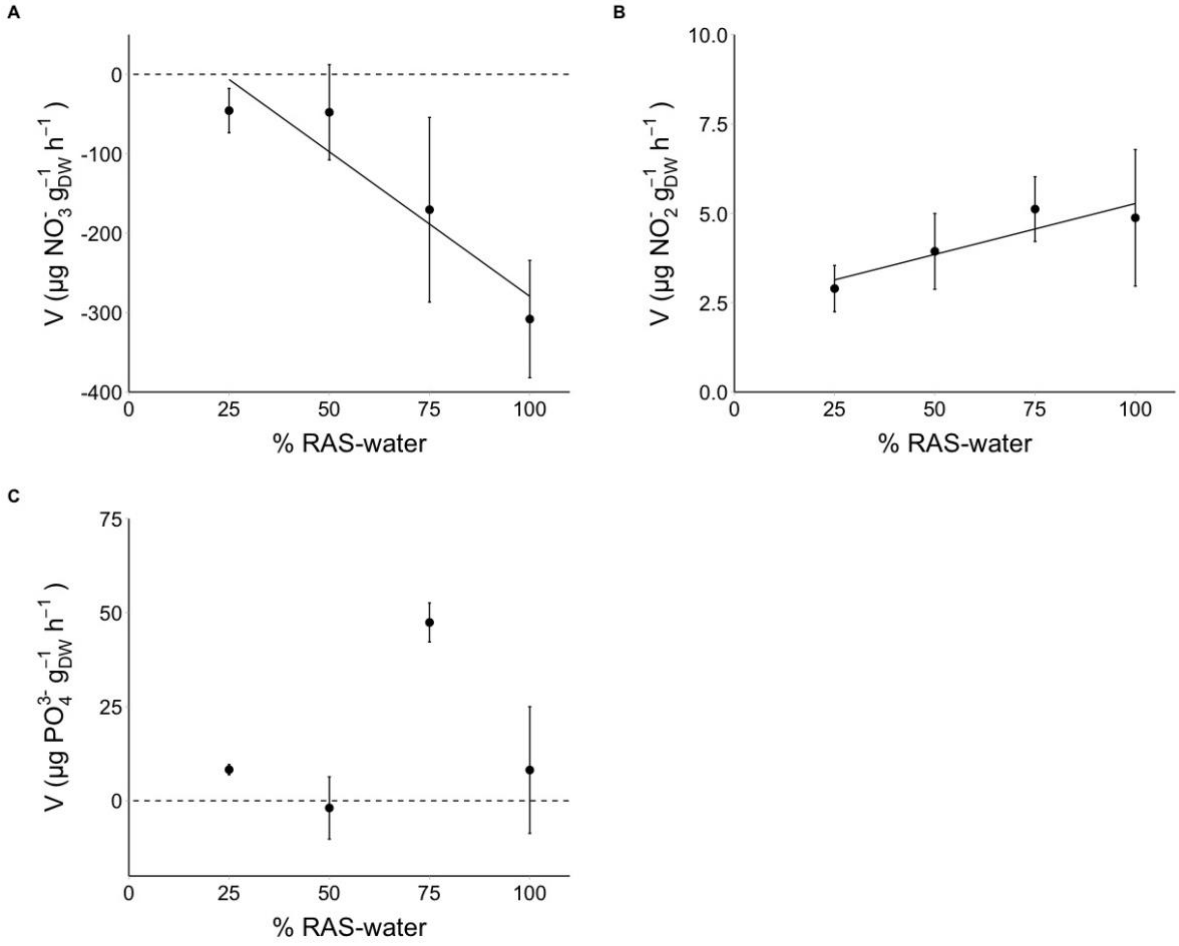


Figure B.1: Uptake rates (V) of A) NO_3^-, B) NO_2^- and C) PO_4^{3-} presented as µg g_{DW}^{-1} h^{-1} for *Ulva* sp. as a function of initial ambient RAS-water concentrations. 100 % RAS-water equals 2213.8 ± 6.7 µg NH_4 L^{-1} ~ 158.1 ± 0.5 µM NH_4. Presented as mean ± SE (n = 4). For A) and B), line represent linear regression through the data. Notice different values on y-axis.

Table B.1: Ammonium uptake rate (V, µg NH_4 g_{DW}^{-1} h^{-1}) values across treatments in Experiment I per time interval. Presented as mean± SE (n = 4).

% RAS	Time interval (min)						
water	0-10	10-20	20-40	40-80	80-160	160-320	320-480
25 %	71 ± 47	51 ± 18	0 ± 0	292 ± 30	325 ± 27	12 ± 2	4 ± 2
50 %	5 ± 79	67 ± 14	1 ± 0	628 ± 56	500 ± 105	56 ± 63	6 ± 6
75 %	-348 ± 132	150 ± 60	2 ± 1	494 ± 93	476 ± 36	73 ± 36	196 ± 17
100 %	54 ± 32	24 ± 29	2 ± 0	737 ± 95	940 ± 81	253 ± 24	160 ± 40

B.2 Uptake Experiment II: Density-gradient

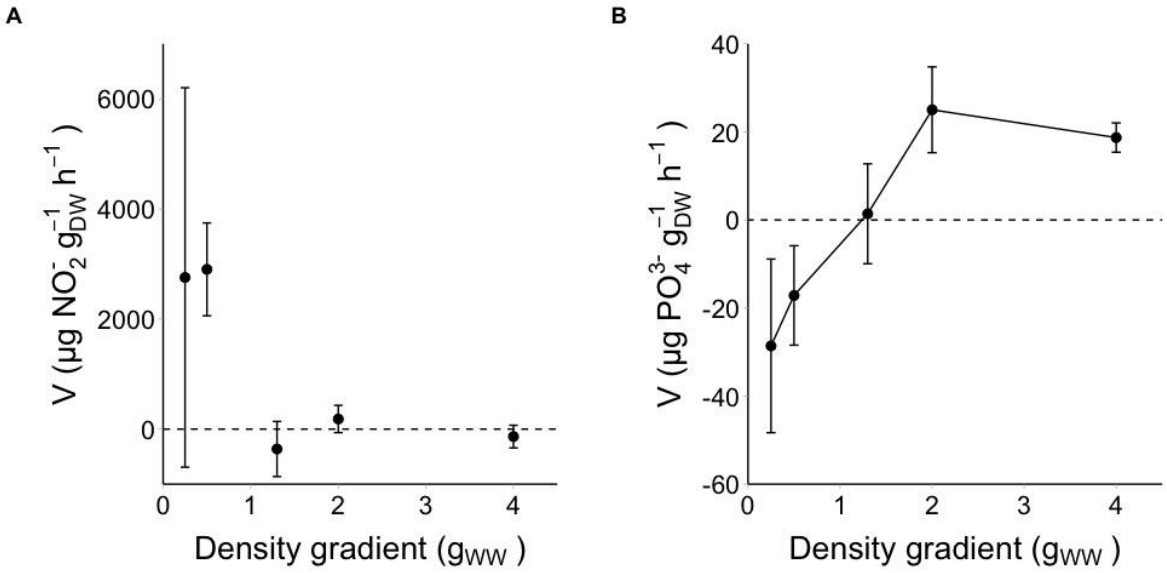


Figure B.2: Uptake rates of A) NO_2^- (V, µg g_{DW}^{-1} h^{-1}) and B) PO_4^{3-} presented as (V, µg g_{DW}^{-1} h^{-1}) for Ulva sp. as a function of initial density (g_{WW}) per 250 mL. Presented as Mean ± SE, n = 4. Notice different values on y-axis.

Table B.2: Ammonium uptake rate (V, µg NH_4 g_{DW}^{-1} h^{-1}) values across treatments in Experiment II per time interval. Presented as mean ± SE (n = 4).

g_{WW}	Time interval (min)							
	0-10	10-20	20-40	40-80	80-160	160-320	320-480	480-600
0.25	50 ± 170	381 ± 139	-32 ± 74	28 ± 41	45 ± 23	166 ± 15	55 ± 2	47 ± 9
0.5	63 ±42	26 ± 64	23 ± 62	87 ± 16	45 ± 16	77 ± 7	23 ± 2	20 ± 1
1.3	44 ± 15	72 ± 50	47 ± 29	60 ± 6	60 ± 3	7 ± 0	2 ± 1	1 ± 1
2	32 ± 20	95 ± 22	78 ± 4	-15 ± 61	57 ± 34	2 ± 0	0 ± 0	1 ± 0
4	62 ± 4	61 ± 16	48 ± 4	31 ± 2	1 ± 0	0 ± 0	0 ± 0	0 ± 0

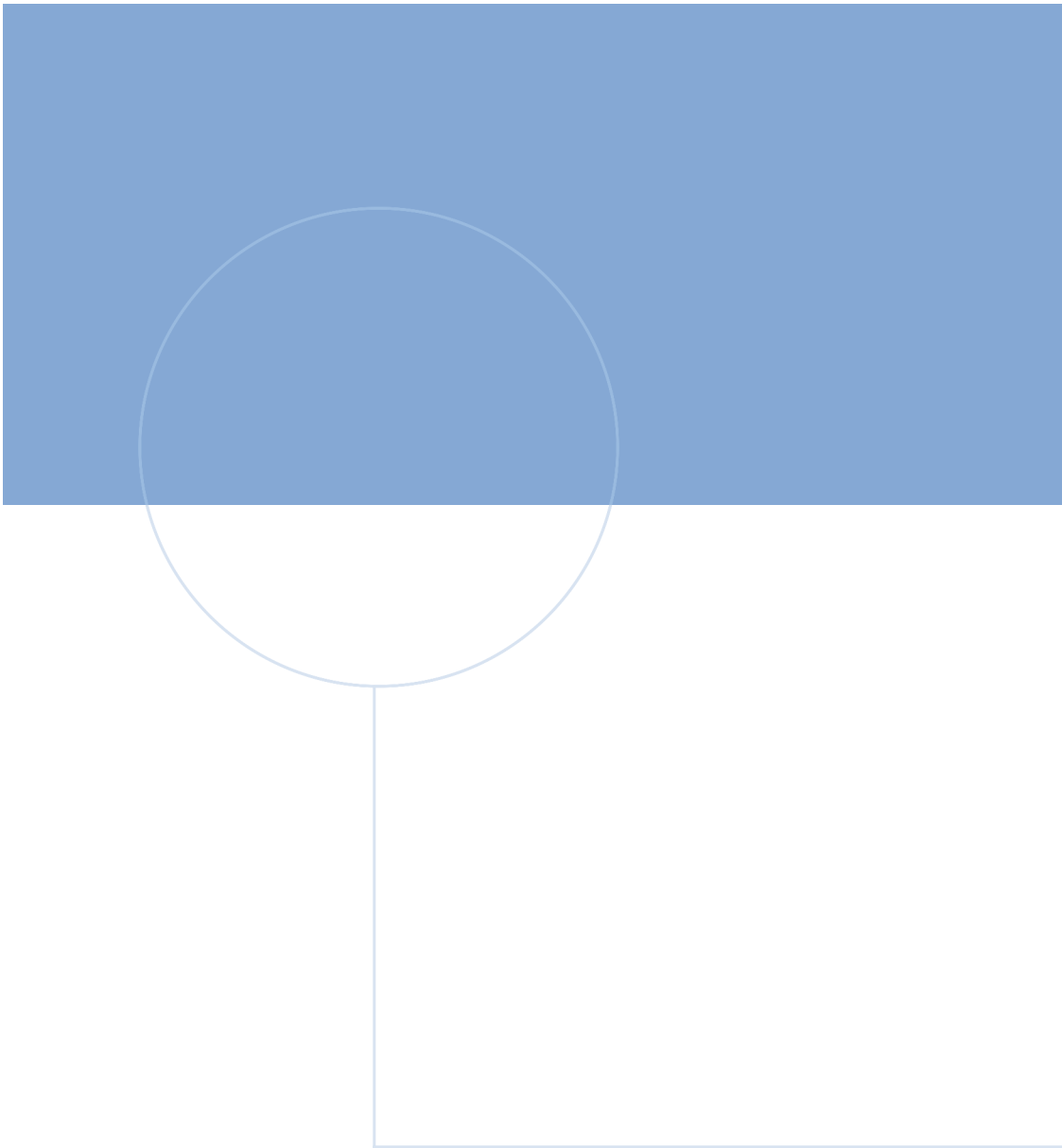
Table B.3: Nitrate uptake rate (V, µg NH_4 g_{DW}^{-1} h^{-1}) values across treatments in Experiment II per time interval up to 80 minutes. Presented as mean ± SE (n = 4).

g_{WW}	Time interval (min)			
	0-10	10-20	20-40	40-80
0.25	85903 ± 87475	-2868 ± 153590	47099 ± 35069	64354 ± 35069
0.5	35288 ± 9430	-46870 ± 26576	74316 ± 34406	-2710 ± 15069
1.3	20916 ± 7692	-6157 ± 12945	18933 ± 10953	11469 ± 7925
2	37858 ± 13919	-39409 ± 9168	1858 ± 5369	8197 ± 3236
4	7401 ± 1181	-3621 ± 1154	2409 ± 2320	1687 ± 1154

Appendix C: Tissue content

Table C.1: Carbon:nitrogen ratios, nitrogen:phosphorus ratios and tissue contents (% of DW) of carbon (C), nitrogen (N) and phosphorus (P) in *Ulva* sp. after exposure to different RAS-water concentrations and densities (per 250 mL) for 8-10 hours. Initial tissue content is given. Values represent means ± SE (n=4 for treatments and n=3 for initial samples).

Treatment	Seawater	25 %	50 %	75 %	100 %	Initial
C:N	7.5 ± 0.2	7.5 ± 0.3	7.5 ± 0.1	7.7 ± 0.2	7.6 ± 0.2	7.2 ± 0.2
N:P	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.0 ± 0.1	0.8 ± 0.2
C (% of DW)	21.2 ± 2.5	26.6 ± 2.4	29.4 ± 1.1	28.0 ± 1.0	24.2 ± 1.1	24.9 ± 3.2
N (% of DW)	2.8 ± 0.3	3.6 ± 0.5	3.9 ± 0.1	3.7 ± 0.3	3.2 ± 0.2	3.4 ± 0.4
P (% of DW)	3.9 ± 0.3	4.8 ± 0.9	3.9 ± 0.4	3.4 ± 0.4	3.4 ± 0.3	4.6 ± 0.6
Treatment	0.25 g	0.5 g	1.3 g	2 g	4 g	Initial
C:N	15.3 ± 1.7	12.8 ± 0.8	14.4 ± 0.5	14.2 ± 0.6	13.3 ± 0.6	12.7 ± 0.4
N:P	0.9 ± 0.2	1.6 ± 0.4	1.2 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	1.2 ± 0.3
C (% of DW)	29.6 ± 1.2	26.5 ± 2.9	26.9 ± 1.9	25.3 ± 3.5	23.5 ± 1.7	25.2 ± 3.0
N (% of DW)	2.0 ± 0.2	2.1 ± 0.4	1.9 ± 0.1	1.8 ± 0.3	1.8 ± 0.1	2.0 ± 0.2
P (% of DW)	2.3 ± 0.4	1.4 ± 0.1	1.8 ± 0.4	2.0 ± 0.2	2.1 ± 0.3	1.7 ± 0.2



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