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Performance of *Ulva* sp. (Chlorophyta) at different stocking densities in Recirculating Aquaculture Systems

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Declaração de autoria de trabalho

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Nikki Schol

Abstract

Aquaculture may be responsible for deterioration of surrounding natural waters, so focus of research in this field to improve sustainability is directed on the recycling of wastewater in cultivation systems. Recirculating Aquaculture Systems (RAS) show promise, but fish production is often limited because of low water quality due to the accumulation of metabolic waste. Water quality may be improved by integrating seaweed as they effectively clean the water by remediating nutrients. This study explored how to optimize RAS by measuring growth and yield performance of *Ulva* sp. and how this relates to water quality. An integration experiment was conducted by adding an *Ulva* filtration unit to outdoor RAS at different fish densities. Seaweed performance was compared by calculating growth rate, biomass yield, and nitrogen removal by biomass of the *Ulva* stocking densities 1, 2, and 3 kg FW m^{-2}. Additionally, water quality parameters were compared, involving biochemical measurements and fluorescent microscopic microbial counts. The results showed that the highest growth rate was measured for *Ulva* stocking density 1 kg FW m^{-2} and the highest biomass and N yield for 2 kg FW m^{-2}. Performance was lower with an increased fish density. The high fish density resulted in lower water quality in the RAS, with a significantly higher abundance of heterotrophic bacteria and bacterivores, a higher level of ammonia, and lower pH. No difference was detected in dissolved oxygen and phytoplankton abundance. These results demonstrate that lower *Ulva* stocking densities show higher performance in IRAS, but more research is required in improving the cultivation environment of *Ulva* to increase its capability to enhance water quality parameters.

Key words: Integrated Recirculating Aquaculture Systems (IRAS), seaweed cultivation, *Ulva* sp., water quality, microbial community

Resumo

A aquacultura é o setor de produção alimentar que mais cresce no mundo, mas devido à alta pressão na procura existe uma preocupação com sua sustentabilidade ambiental. Um dos problemas é a eutrofização das águas naturais devido aos efluentes ricos em nutrientes, com as consequências prejudiciais que dai resultam. É essencial desenvolver estratégias para reduzir os resíduos metabólicos na aquacultura, e sistemas (semi)fechados como os Sistemas de Recirculação de Aquacultura (RAS) que oferecem grande potencial para soluções mais sustentáveis, pois permitem o cultivo de alta densidade de peixes em ambiente controlado.

Os principais desafios com o RAS estão relacionados com as questões de rentabilidade e eficiência. Isso advém da troca reduzida de água o que causa à acumulação de metabólitos de peixes e bactérias, fatores inibidores de crescimento como cortisol dos peixes e metais pesados da ração. Em particular, a toxicidade da amónia leva a uma produção reduzida de peixes, pois inibe o crescimento causando danos oxidativos, danos físicos, doenças ou até mesmo a morte. Além disso, altas concentrações de nitrito, nitrato e fosfato afetam negativamente os peixes. Tanto o fitoplâncton como a comunidade bacteriana podem dar indicação da qualidade da água, pois estes microrganismos respondem rapidamente às mudanças na disponibilidade de nutrientes. As macroalgas podem remover efetivamente nutrientes da água numa abordagem ecológica. As macroalgas têm um elevado potencial para transformar resíduos em coprodutos de valor comercial quando integrados em sistemas de aquacultura, mas este recurso é ainda relativamente inexplorado. Uma possível abordagem sustentável para a gestão de efluentes é a Aquacultura Multitrófica Integrada (IMTA) onde espécies de diferentes níveis tróficos são integradas no mesmo sistema. Como a produção de peixes está, em parte, relacionada com a qualidade da água a presença de micro-organismos nocivos, a biofiltração pelas macroalgas melhora a saúde ambiental ao equilibrar os parâmetros de qualidade da água. As algas do género *Ulva* (filo Chlorophyta) podem ser um recurso sustentável para a indústria de rações e alimentos entre muitos outros, bem como representar uma alternativa de biofiltração, no entanto, o desempenho de crescimento de *Ulva* em RAS revelou-se altamente variável em diferentes estudos. Para desenvolver um RAS integrado com macroalgas (IRAS) economicamente viável, é necessário determinar a densidade de cultivo ideal para uma maior produção de biomassa e absorção de azoto, bem como a influencia da densidade de peixe nestes fatores.

Os objetivos específicos deste estudo foram (a) determinar a densidade ótima de cultivo de *Ulva* para uma maior produção de biomassa, (b) investigar como a produção de algas

influencia parâmetros de qualidade da água, e (c) comparar como a densidade de peixes influencia o desempenho das macroalgas.

Para responder a essas perguntas, foram usados dois IRAS piloto. Um com alta densidade de peixes (IRAS High-F) e outro baixa densidade de peixes (IRAS Low-F) de modo a comparar como uma carga de nutrientes diferente influência o desempenho da *Ulva* sp. O ensaio foi iniciado com 20 e 10 kg m^{-3} de dourada, *Sparus aurata*, mas após duas colheitas essas densidades foram reduzidas para 10 e 5 kg m^{-3}, respetivamente, porque os níveis de amónia excederam 1 mg L^{-1} no IRAS High-F. Portanto, os dados foram separados no Ciclo 1 (colheitas 1 e 2) e Ciclo 2 (colheitas 3, 4 e 5). O ensaio experimental teve lugar, no exterior, nos meses de abril e maio. A temperatura da água foi controlada na medida do possível. Cada sistema incluiu as densidades de *Ulva* 1, 2 e 3 kg FW m^{-2} em triplicado para encontrar a densidade de cultivo ideal para produção de biomassa e biorremediação. A taxa de crescimento específico (SGR), produção de biomassa (g FW m^{-2}) e biofiltração de azoto (g N m^{-2}) da *Ulva* foram determinados medindo o peso fresco, colhendo subamostras e secando-as no forno para calcular a relação linear para converter valores de peso fresco, peso seco e conteúdo de carbono-azoto (C-N). Além disso, foram feitas análises microscópicas de amostras de tecido *Ulva* para verificar o estado reprodutivo, uma vez que isso afeta o seu desempenho. Todas as semanas foram colhidas amostras de água para medir os níveis de nutrientes (nitrato, nitrito e amônio) nos dois sistemas. Como o Ciclo 2 teve um melhor desempenho em comparação com o Ciclo 1, resumimos apenas os dados do Ciclo 2 seguidamente.

A biomassa de *Ulva* foi medida uma vez por semana para calcular a taxa de crescimento, produção de biomassa e biofiltração de azoto. A baixa densidade de peixes apresentou maiores taxas de crescimento (M = 26,7% d^{-1}) e produção de biomassa de *Ulva* (M = 1064,8 g m^{-2} d^{-1}) do que a maior densidade (M = 21,4% d^{-1} e 770,4 g m^{-2} d^{-1}). No Ciclo 2, o desempenho do IRAS Low-F foi mais alto que no Ciclo 1, apesar da redução em amónia, enquanto o IRAS High-F permaneceu semelhante. Estes resultados podem ser devidos aos seguintes fatores: (1) a carga ideal de nutrientes para *Ulva* foi ultrapassada no IRAS High-F e inibiu o crescimento, (2) a alta carga de nutrientes no IRAS High-F promoveu o crescimento de microrganismos causadores de doenças que stressaram a *Ulva*, (3) outros parâmetros ambientais, como carbono disponível, favoreceram a *Ulva* no IRAS Low-F.

As densidades de *Ulva* tiveram um desempenho produtivo diferente dependendo do tratamento de densidade de peixes. O SGR foi maior na densidade *Ulva* 1 kg m^{-2}, e foi significativamente maior no IRAS Low-F (M = 37,6% d^{-1}) do que High-F (M = 25,5% d^{-1}).

Não houve diferença significativa na produção de biomassa no IRAS Low-F, mas no IRAS High-F foi significativamente maior para densidade de *Ulva* 2 kg m^{-2} (*M* = 1039,4 g FW m^{-2} d^{-1} / 139,7 g DW m^{-2} d^{-1}) do que para 1 kg m^{-2} (*M* = 704,3 g FW m^{-2} d^{-1} / 94,6 g DW m^{-2} d^{-1}). Também não encontrámos efeito significativo na densidade de *Ulva* em relação ao conteúdo de N. Embora o conteúdo de N no tecido *Ulva* tenha sido menor em IRAS Low-F (*M* = 2,56 g N m^{-2} d^{-1}) do que High-F (*M* = 2,94 g N m^{-2} d^{-1}), a remoção de N foi significativamente maior para IRAS Low-F (*M* = 4,28 g N m^{-2} d^{-1}) do que High-F (*M* = 3,55 g N m^{-2} d^{-1}) devido à maior produção de biomassa de Ulva. Acreditamos que, ao otimizar o crescimento e produtividade da *Ulva*, esta torna-se mais capaz de bioremediar a água.

Para avaliar os parâmetros de qualidade da água, foram medidos oxigénio dissolvido (DO), pH e temperatura da água todos os dias pela manhã e à tarde. Enquanto o DO diminuiu durante o dia, o pH aumentou. Amostras de água para análise de amónia, nitrato, nitrito e ortofosfato foram recolhidas uma vez por semana, no entanto, os resultados das análises de nutrientes dissolvidos ainda não estão disponíveis. Presumivelmente fosfatos e nitritos estariam extremamente elevados em IRAS High-F, inibindo o crescimento de *Ulva*. Os níveis elevados de nitrogênio também se refletiram no maior teor de N no tecido de *Ulva*. Esta suposição é apoiada pelas contagens microscópicas que mostraram uma alta abundância em cianobactérias, diatomáceas, protistas bacterivores e bactérias heterotróficas. Estes constituem indicadores de água relativamente eutrofizada. As contagens também foram significativamente maiores no IRAS High-F do que Low-F. No geral, podemos dizer que o IRAS High-F teve uma qualidade de água muito pior do que Low-F, e não foi observada melhoria significativa nos parâmetros medidos em ambos os IRAS durante a experiência.

Os resultados sugerem que *Ulva* é uma opção promissora como unidade de biofiltração, uma vez que as altas taxas de crescimento contribuíram para a remoção de quantidades consideráveis de nutrientes na água. Para investigação futuras, recomenda-se otimizar o ambiente de cultivo da unidade *Ulva*, estudando como melhorar sua capacidade de manter boa qualidade da água com maiores densidades de peixes tornando IRAS mais sustentável em maior escala.

Palavras-chave: Sistemas Multitróficos Integrados de Aquacultura em Recirculação (IRAS), cultivo de macroalgas, *Ulva* sp., qualidade da água, comunidade microbiana

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List of abbreviations

AGR	Average Growth Rate
BGI	Blue Growth Initiative
d	Day
DIN	Dissolved Inorganic Nitrogen
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DW	Dry Weight
EPPO	Estação Piloto de Piscicultura de Olhão
FCR	Feed Conversion Ratio
FW	Fresh Weight
IPMA	Instituto Português do Mar e da Atmosfera
IAA	Integrated Aquaculture/Agriculture
IMTA	Integrated Multitrophic Aquaculture
I-RAS	Integrated Recirculating Aquaculture System
M	Mean
Md	Median
POM	Particulate Organic Matter
RAS	Recirculating Aquaculture Systems
SGR	Specific Growth Rate
SDGs	Sustainable Development Goals
TAN	Total Ammonia Nitrogen
TSS	Total Suspended Solids
UALG	University of Algarve

1. Introduction

The theoretical considerations that inform this study include the water quality in aquaculture, recirculating systems, and the potential of seaweed integration.

1.1 Aquaculture and water quality

Aquaculture is a growing industry worldwide, but faces challenges regarding environmental sustainability (FAO, 2019). Sustainable development of aquaculture should be achieved by utilizing fewer resources like space, water, and energy so it limits its impact on the environment, while promoting food security and economic growth. These were themes that are part of the Sustainable Development Goals (SDGs) from the international sustainability agenda for 2030 (FAO, 2017). However, intensification of aquaculture can deteriorate water quality, which is a concern for the health and welfare of the cultured fish (Matos et al., 2017). Gilthead seabream *Sparus aurata* is an important fish species in European aquaculture and is mainly cultured in Mediterranean countries, with production systems ranging from semi-intensive production in earth ponds, to highly intensive land-based systems, such as tanks, raceways, and sea cages (Basurco et al., 2011). Water quality in pond systems rely on natural processes by the biological community and recycling capacity is limited and unstable due to unpredictable fluctuations in microbial dynamics, while nutrient enrichment to the environment occurs by water renewal and drainage and seepage to groundwater (Hargreaves, 1998). Flow-through systems include raceways, tanks and cages, and their metabolic waste is directly discharged to the surrounding environment by a continuous exchange flow. Feed management and wastewater treatment techniques are necessary to avoid eutrophication, but it is still difficult to manage the discharge of waste materials because of the high volumes of effluent with low nutrient concentrations (Verdegem, 2013). Recirculating systems on the other hand considerably reduce the discharge of nutrients to the environment and hereby offer a more sustainable and environmentally friendly solution than conventional marine aquaculture (Ahmed & Turchini, 2021). However, this industry deals with profitability and efficiency issues due to high competition in this market (Llorente et al., 2020). These issues could be tackled by developing strategies to improve recirculating water quality.

1.2 Nutrient loading and effects

A high nutrient load in the water promotes toxicity, organic enrichment and sedimentation which often leads to undesirable alterations in function and structure of the ecosystem (Gabric & Bell, 1993). Waste materials in cultivated water are mainly composed of faeces and uneaten food and can be found in forms like nitrogen (N) and phosphorus (P), particulate and dissolved organic matter (POM and DOM), total suspended solids (TSS), and specific inorganic and organic compounds. It also includes chemicals that are used to treat infections and pathogens. All these materials contribute to the solids in the water as well, increasing water turbidity (Dauda et al., 2019). In production systems, a high nutrient-load in the system increases maintenance costs and reduces economic output. For example, Seo et al. (2020) observed that the accumulation of inorganic nitrogen compounds induce oxidative stress and inhibit innate immunity in cultivated fish, factors that lead to a reduced productivity. To avoid issues like this, international regulations of water quality parameters are established (Table 1.1).

Table 1.1: Water quality parameters for fish cultivation (FOS, 2014).

Water quality parameter	Quantity
Ammonium (NH_4)	≤ 1.0 mg L^{-1}
Nitrate (NO_3^-)	≤ 15.0 mg L^{-1}
Nitrite (NO_2)	≤ 1.0 mg L^{-1}
Phosphate (PO_4)	≤ 0.2 mg L^{-1}
Dissolved oxygen (DO)	≥ 5.0 mg L^{-1}
CO_2	< 2.0 ppm

1.2.1 Ammonia toxicity

In aquaculture water nitrogen is primarily found as ammonia, a product from fish respiration and organic matter decomposition. Fish have a high sensitivity to high ammonia levels because exposure increases the amount of ammonia in their blood. This can lead to ammonia toxicity with a direct impact on growth, disease resistance, oxidative damage, physiological problems and even mortality (Lemarie et al., 2004). Ammonia can be found in the forms NH_3 and NH_4^+ and the combination is referred to as total ammonia nitrogen (TAN). Ammonium, NH_4^+, can be harmful to the fish, and the metabolite unionized ammonia, NH_3, can already be toxic at low concentrations (Eshchar et al., 2006).

1.2.2 Nitrification and denitrification

Ammonia is oxidized to nitrite and then nitrate by bacterial activity called nitrification, it can be further reduced to nitrogen gas by denitrification and hereby removed from the water. The pH is an important factor that influences decomposition processes of ammonia by altering dissolved CO_2 and NH_3 concentrations in the water. Nitrifying bacteria require a pH between 7 and 8.5 (Hargreaves, 1998). A pH higher than 8.6 causes nitrite accumulation while lower than 7.0 reduces denitrification rate. Nitrite has a higher risk of toxication because it can be actively taken up through the gills, leading to high concentrations in the body. This causes multiple physiological effects. It disrupts processes that include ion regulation, respiration, excretion, and endocrine function. Nitrite is particularly toxic because it competes with chloride to bind to hemoglobin so it loses its capacity to carry oxygen (Jensen, 2003). The most important factors that influence nitrite toxicity to fish are water quality, temperature, chloride concentration, length of exposure, fish species, age, size, and individual susceptibility (Kroupova et al., 2005).

High nitrate concentrations can be toxic because nitrate reacts with hemoglobin, and this causes a shortage of oxygen in the fish which leads to death (Camargo et al., 2005). Nitrate toxicity increases with elevated levels and exposure times, and decreases with environmental adaptation, water salinity, and increased body size. Although many research found no noticeable effects of high nitrate levels on fish health, they did observe a reduced maximum oxygen uptake and decreased growth rate (Isaza et al., 2018; Seo et al., 2020). Strauch et al. (2019) found similar results for phosphate (PO_4-P). This is the main form of P, and a natural product of waste breakdown. Denitrification helps with controlling phosphate levels (Barak et al., 2003).

1.2.3 pH level

The pH is a very important variable in water quality as H^+ affects many reactions, and thereby affects nearly every water treatment function. Respiration processes produce H^+ and CO_2, both decreasing the pH, making the water more acidic. Photosynthesis removes these compounds, increasing the pH. Carbon reacts in water, forming an acidic solution by forming H_2CO_3. A higher pH increases the alkalinity of the water. Alkalinity is the solution´s ability to react with acid and is expressed as mg L^{-1} calcium carbonate ($CaCO_3$). It protects the water from large daily swings in pH that would otherwise happen due to photosynthesis during the day and respiration processes during the night. Additionally,

alkalinity increases photosynthesis as bicarbonate can be taken up by many phytoplankton species and aquatic plants. For most aquatic organisms the optimum pH ranges between 6.5 and 8.5. (Boyd, 2015; Xiao et al., 2021)

1.2.4 Oxygen level

The environment of the fish production system is very important for optimum growth, survival, feed conversion and profits. Dissolved oxygen (DO) concentration is the most important environmental variable for fish species, and in particular seabream is sensitive to hypoxia (< 2 mg O_2 L^{-1}) which negatively affects growth and feed conversion, and ultimately death (Abdel-Tawwab et al., 2019; Pichavant et al., 2001). When there is a high level of dissolved organic carbon in the water due to fish waste and uneaten food together with high nitrite-nitrate levels, the growth of heterotrophic microorganisms is promoted. Denitrification to support their metabolism contributes to the depletion of dissolved oxygen in the water and the production of CO_2 (Van Rijn et al., 2006). Increased CO_2 also causes toxicity because less internal CO_2 produced by fish metabolism can be transferred from the gills to the water. This results in reduced oxygen levels in the fish. The level of CO_2 in the water depends on alkalinity, water pH, and temperature (Piedrahita, 2003). High DO can be maintained by water oxygenation, but this also promotes processes that increase nutrient fluxes. By using biofiltration these effects can be mitigated.

1.4 Microbial community

The ecological health and quality of marine environments can be defined by determining phytoplankton community composition (Galvão et al., 2012). Phytoplankton are photosynthetic microorganisms that live in the water column and contribute greatly to maintain a balanced ecosystem (Hoppenrath et al., 2009). Both phytoplankton and bacterial community structure, growth and biomass rapidly respond to changes in nutrient availability (Barbosa et al., 2010; Galvão et al., 2008). The phytoplankton community, in particular cyanobacteria, and ammonia concentration play a significant role in DIN removal, as ammonia is the preferred N form for phytoplankton, and cyanobacteria play a significant role in N fixation. The main factors that affect phytoplankton growth are temperature and the nutrients NO_3, NH_4, and PO_4. Phytoplankton takes up these dissolved nutrients by photosynthesis and production (Hargreaves, 1998).

Phytoplankton diversity can give an indication of water quality. For example, diatoms grow well in nitrate rich water, while dinoflagellates grow well in ammonium rich water (Glibert & Terlizzi, 1999). Diatoms are desirable in aquatic environments for a high abundance is associated with a high bacterial diversity that promotes remineralization processes (Boyd, 2014). Dinoflagellates on the other hand are mostly associated with deteriorating water quality because some species produce toxins that can cause mortality by poisoning the fish, as well as reducing bacterial production and variety (Camacho et al., 2007). Many photosynthetic nano- and dinoflagellates are mixotrophic and can dominate the phytoplankton community as they can both perform photosynthesis as grazing on bacteria (Camarena-Gómez et al., 2021; Laybourn-Parry & Parry, 2000). Since bacterioplankton exploit the dissolved organic carbon (DOC) pool, they are abundant in fish culture. Understanding the factors that influence microbial dynamics in aquaculture systems can be valuable tools to enhance water quality.

1.5 Recirculating Aquaculture Systems (RAS)

Sustainability in aquaculture can be improved by using closed systems like recirculating aquaculture systems (RAS) that are based on water treatment and recycling. This allows the intensification of tank production while producing high quality fish by reducing risks due to disease, parasites, and climatic factors (Martins et al., 2010). Although the higher biomass produces more waste materials, this system also creates the potential to effectively treat effluent (Piedrahita, 2003). The water is purified by different filtration mechanics before it recirculates into the system and hereby generates a lower environmental impact by minimizing water usage and effluent release to the environment (Martins et al., 2010). There is an increased interest in RAS, because its technology can effectively overcome limitations like location, environmental impacts, and water availability.

1.5.1 RAS and water treatment

RAS is highly effective in removing organic matter, suspended solids, and phosphorus from the system. Water is purified by mechanical filters to remove solid particles, and biological filters to remove ammonia by nitrifying bacteria. Most commercial RAS have no nitrate removal unit because nitrate is relatively harmless to aquatic species. Suspended solids are primarily fecal matter and are continuously removed from the system by a filter screen (Dauda et al., 2019). Biofilters, including bioballs, are useful for water quality management in RAS because they promote the attachment of microbes that

deal with phosphorus and dissolved inorganic nitrogen (DIN) waste by complete nitrification, which optimizes water quality and reduces the need to exchange water (Díaz et al., 2012). Even though the cultured water is filtered to improve the water quality in the system, the recycling of water continuously accumulates metabolic products of bacteria and fish. The high stocking density can lead to nutrient overloading, and this can cause stress, loss of productivity, and even mortality to the cultured fish (Martins et al., 2010). It is important to remove solid wastes from the system because it can clog the gills of the fish which leads to death (Akinwole et al., 2016). The main challenge of RAS is that microbial activities in RAS biofilters are not able to completely remove solid and dissolved organic wastes. Some nutrients are relocated instead of recycled. For example, inside the biofilters and solids removal systems organic matter can accumulate and decompose (Piedrahita, 2003). For this reason, it is important to develop effective water treatment technology to remove N and P from the water.

1.5.2 Multitrophic approach

In natural systems the excretion of fish is mostly absorbed by associated algae, so an efficient way to trap inorganic nutrients is to grow phytoplankton and seaweeds integrated in aquaculture systems, also known as ecosystem-based management (Roleda & Hurd, 2019). Examples are integrated multitrophic aquaculture (IMTA) and integrated aquaculture/agriculture (IAA) systems. These systems offer sustainable approaches for effluent management, water recycling, and biological treatment. By combining species of different trophic levels in the production system, nutrients are recycled efficiently (Buchholz et al., 2012). Another advantage of IMTA systems is that different species can be cultured in separate spatial units (Shpigel et al., 2018). Cunha et al. (2019) already observed that a multitrophic system in earthen ponds enhances water quality and higher fish biomass production, increasing the profitability for the farmer. They evaluated the role of fish, filter feeders, phytoplankton and seaweed in a pond system and found a higher and more stable DO, an increase in transparency, and the capture of excess of nutrients. Although there are many benefits of holistic approaches, such as enhanced productivity and nutrient extraction, these systems are for farmers often too complex for easy management. Likewise, there must be a compensation for the extra costs due to the space and pumping requirements for the treatment units (Verdegem, 2013). Therefore seaweed aquaculture is proposed as a sustainable and cost-effectively biofilter, because it offers a supply of feed, food and high value biochemical products (Buschmann et al., 2017;

Chopin et al., 2001). In closed systems this offers a less expensive method to improve water quality than bacterial and mechanical water treatments (Schuenhoff et al., 2003). To implement seaweed cultivation in RAS in the most beneficial and practical way, further study is required about seaweed integration strategies and seaweed performance in this system.

1.6 Seaweeds as biofilters

In fish production, the reduction in growth and development, and mortality can be attributed to water quality and the presence of harmful micro-organisms. Water quality in production systems can be improved by using seaweeds which are relatively easy to harvest that have a great potential to absorb significant amounts of dissolved organic and inorganic nutrients by effectivity taking up phosphate, nitrate and ammonium for growth and photosynthesis. Seaweed biofiltration counters microbial and fish heterotrophy, balancing nutrients, CO_2, oxygen and pH in the system (Salvaterra et al., 2021). Studies have shown that seaweed biofiltration can outperform bacterial processes because they keep DIN levels consistently low, and pH and DO levels steadier (Cahill et al., 2010), and can reduce mortality rate and accumulation of metals in the fish when applied in RAS (Metaxa et al., 2006).

1.6.1 Seaweed cultivation

To effectively use seaweed as biofilter to limit nutrients in cultivated water, it is important to explore the performance of seaweed species in this environment. The principal criteria to select seaweed species are a high growth rate and biomass yield, low energy and nutrient requirements, ease of management, and intrinsic properties like moisture content (Bruhn et al., 2011). Season, stocking density, and nutrient load of the cultivated water are important factors for specific growth and maximum uptake rates (Troell et al., 2003). Optimal seaweed density will maximize cultivation yield and can be enhanced by water mixing to exploit carbon fixation rates and photon capture. Seaweed performance can decrease when density causes self-shading and nutrient depletion, and the chance of disease will increase (Neori et al., 2004). Although specific growth rate is inversely related to density (Viaroli et al., 1996), Xiao et al. (2019) showed that seaweeds can grow in high densities and achieve high growth rates in environments with high ammonia concentrations and irradiance.

Other factors that influence performance are temperature, light intensity, salinity, water motion, competition for resources, losses to pathogens and grazers, and reproductive state (Roleda & Hurd, 2019). Reproduction can limit growth rate because the transition from vegetative state to sporulation stage reduces photosynthetic performance that can be caused by self-shading of spores (Park, 2020; Roleda & Hurd, 2019). In *Ulva*, as a dark green color might indicate an increased photosynthetic activity, sporulation can be recognized by the appearance of white or transparent tissue and growth inhibition before releasing the reproduction cells (Dan et al., 2002). In temperate regions, longer days can reinforces the vegetative growth phase, while shorter days can trigger reproduction, which results in lower growth rates (Lüning et al., 2008). Also temperature fluctuations, temperature shock (5°C), salinity, and thallus fragmentation can initiate reproduction (Balar & Mantri, 2020; Kalita & Titlyanov, 2013). A challenge in seaweed cultivation is the growth of algal epiphytes that both compete for nutrients and often use the cultured seaweed and/or tank surface as substrate. Methods to control epiphyte growth are frequent harvesting of the cultured seaweed, water flow, water level changes, and high stocking density (Neori et al., 2004). To improve profitability of seaweed integration in aquaculture, it is important to study how to optimize seaweed performance in these systems.

1.6.2 *Ulva* performance

Ulva (Chlorophyta) is a genus of green algae with a cosmopolitan distribution with a high commercial interest (Silva et al., 2013), and has been identified to be an ideal species to biofilter cultivated water from fish farms because its high photosynthetic rates results in a high biomass production and high ability to take up dissolved nitrogen when continuously supplied with nutrients rich water (e.g. Aníbal et al., 2014; Ben-Ari et al., 2014). Anyhow, the performance of *Ulva* is highly variable when looking at different research. For example, Fotedar (2016) used a seaweed as biofilter to overcome nutrient overloading and animal welfare problems in RAS, and concluded that the biofilter capacity and optimum stocking density of seaweeds depends on surface area of the biofilter unit, hydraulic loading (L d^{-1} cm^{-2}), and turnover time. Another challenge is that data is difficult to compare because of the use of different functions of fresh weight (FW), dry weight (DW), or surface area to volume.

Most studies investigated the performance of the species *Ulva lactuca.* They found that N uptake rates can reach up to 74-80% when supplied with N rich water from aquaculture

effluent (Adharini et al., 2021; Shpigel et al., 2019). Sode et al. (2013) measured the performance at different NH_4^+ concentrations obtained from wastewater sludge. They achieved maximal growth rates of 54.5% FW d^{-1} with 50 µM NH_4^+. The nutrient removal rates found a maximum of 22.7 mg N g DW^{-1} d^{-1} and 2.7 mg P g DW^{-1} d^{-1} with 80 and 89 µM NH_4^+, respectively. Shpigel et al. (2018) observed that the production ranged from 70 – 350 g m^{-2} d^{-1} from winter to summer, respectively, and Al-Hafedh et al. (2015) shows that high flow rate (7.5 L min^{-1}) favors the growth of *Ulva lactuca*. Viaroli et al. (1996) estimated that the critical biomass threshold of *Ulva rigida* is 1 kg ash free DW m^{-3}. They found that after this point, active production can switch to rapid decomposition due to biomass density, self-shading, and oxygen depletion. When evaluating the nutrient bioremediation and biomass production potential of *Ulva* sp. with different stocking densities in RAS, a sustainable water treatment technology can be developed with economic advantage compared to RAS without a seaweed biofiltration unit.

1.7 Study aim

The aim of this work was to develop a holistic approach to reduce the nutrient load of recycled water by seaweed production in RAS aquaculture. To develop a productive, efficient, disease-free, and biologically secure system it is important to explore how the biological filtration system reacts on nutrient input, since this is not as easily controlled as the mechanical filtration systems. To cope with this objective, *Ulva* sp. was integrated to form an integrated recirculating aquaculture system (IRAS) which held gilt-head seabream *(Sparus aurata*) at two different densities. The capacity of *Ulva* sp. to produce biomass and simultaneously improve water quality in IRAS with different fish densities was assessed. Additionally, *Ulva* was compared at stocking densities 1, 2, and 3 kg m^{-2} to determine which density performs best in tested IRAS. The culture was conducted during April and May in South Portugal.

We hypothesized that the IRAS with the higher fish density would give a higher growth rate and yield because of the larger supply of nitrogen in the system. We also postulated that the IRAS with higher *Ulva* biomass would show improvement in water quality. Lastly, we speculated that if resources are limiting in the seaweed tanks, there will be a difference found in performance parameters by *Ulva* between the two experimental IRAS and stocking density treatments.

This study investigates how RAS can be optimized by using seaweeds as biofiltration unit and intends to improve the knowledge of the ecological effects and economic potential of cultivating commercially interesting seaweed species in closed systems which might encourage farmers to invest in more sustainable aquaculture practices.

2. Research methodology

This research was carried out for a total of 5 weeks from 21st of April to 25th of May 2022 in South Portugal (37° 03´N; 07° 82´W) at Estação Piloto de Piscicultura de Olhão (EPPO), a research facility from the Instituto Português do Mar e da Atmosfera (IPMA). Two outdoor IRAS holding gilthead seabream *Sparus aurata* in two different densities were compared using *Ulva* sp. as seaweed biofiltration unit. During the experiment water quality of the IRAS was assessed, including chemical and microbial variables. In addition, the performance of *Ulva* considering growth rate, biomass yield, and N removal were compared at three different stocking densities.

2.1 Experimental setup

The IRAS were located outdoors and exposed to natural light and a Mediterranean climate. Water inflow derived locally from Ria Formosa and was filtered through sand filters before it entered the system. The IRAS consisted of a fish unit, protein skimmer (organic compounds removal), solids settling compartment, bacterial biofiltration unit (denitrification, ammonia removal), oyster shells filtration unit and sponge mats (suspended solids removal), and seaweed unit (dissolved nutrients removal). In the filtrated water unit, water was pumped to the seaweed biofiltration unit, water temperature was controlled, and pure oxygen was added (Figure 2.1 & Figure 2.2).

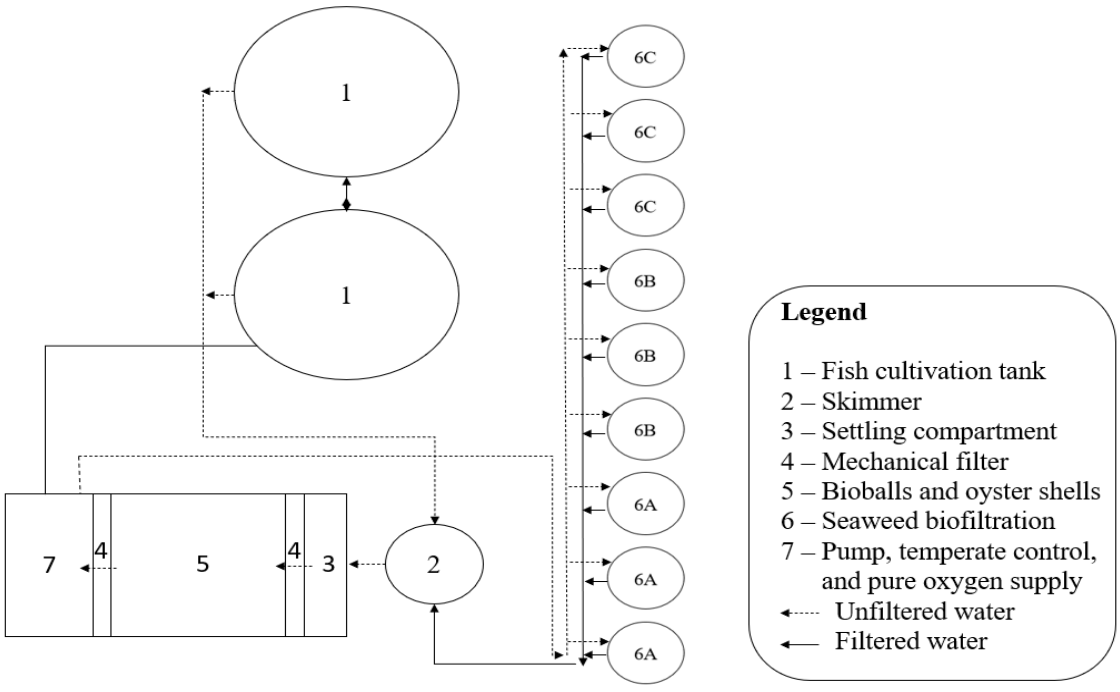

Figure 2.1: Schematic view from above of experimental IRAS showing all the functional parts of the system; A, B & C indicate different Ulva stocking densities with a randomized location each week. Functional parts 1, 4, 5, and 7 were protected from sunlight with a cover.

Figure 2.2: Side view of the two experimental IRAS.

2.1.1 Fish unit

The fish unit consisted of two 3000 L (Ø210 cm) fish tanks that started with a fish stock density of 20 and 10 kg m^{-3} of juvenile seabream for harvest 1 and 2, but fish density was lowered to 10 and 5 kg m^{-3} for harvest 3, 4, and 5 to avoid harmful ammonia levels to the fish. The two experimental IRAS are referred to as IRAS High-F and IRAS Low-F, respectively. The fish were hatched and reared by EPPO in standard rearing conditions. All culture tanks received continuous aeration and water circulation. The water flowed through a temperature control unit and was kept around 20-25°C. Turnover rate per fish tank was kept at 1.67 h^{-1}. The fish tanks received an equal quantity of water exchange in both IRAS whenever one of the fish tanks exceeded ammonia levels of 1.0 mg L^{-1}. Water quality was controlled by feeding a lower feed ration to the fish than recommended by the feed supplier to avoid the accumulation of uneaten feed in the fish tanks. The fish were hand-fed four times a day with pelleted commercial feed (AquaSoja standard orange 6) between 9:30 and 16:30 hours at a feeding rate of 0.98% of the average body weight for the first 3 harvests. As a higher temperature increases the energy demand of the fish by stimulating the activity, growth, and metabolism, food ration was raised to 1.49% for harvests 4 and 5.

2.1.2 Seaweed biofiltration unit

The seaweed unit consisted of nine 200L tanks (Ø55 cm), with three different stocking densities in triplicate. Aeration was situated at the bottom of the tank to facilitate tumble

culture, and an inflow and outflow filter were situated at water surface to maintain water level (Figure 2.3A). Flow rate per seaweed tank started at 720 L h^{-1} in the first week but was reduced to 514 L h^{-1}. As the seaweed tanks kept overflowing, the turnover rate was adjusted to 450 L h^{-1} during harvest 3 and was determined to be the highest possible rate in this experimental system.

Two weeks prior to the experiment, *Ulva* sp. in healthy condition with bright green ruffled blades was collected from the earthen ponds at EPPO and washed in an outside tank with clean salt water to remove epiphytes, debris, and encrusting organisms (Figure 2.3B). *Ulva* was kept at this location with continuous aeration and water flow until the experiment started. At the beginning of the experiment, the seaweeds were removed from the tanks, excess of water was removed by placing the seaweeds on a mesh tray for approximately 30 min, and fronds of comparable sizes were selected to stock the seaweed tanks with a fixed FW to form the three stocking density treatments 1, 2, and 3 kg m^{-2} in triplicate. In the experimental tanks this was calculated to be 237, 474, and 711 g for treatment 1, 2, and 3, respectively. The *Ulva* performance in the two different IRAS and between stocking densities were compared separately.

Figure 2.3: A = Overview seaweed biofiltration unit tank showing inflow tube and outflow filter; B = Cleaned Ulva sp. from the pond in EPPO on mesh tray.

2.2 Water quality

2.2.1 Environmental factors

Water quality parameters DO (Sinergia Oxymeter) and pH (Fisherbrand accumet AB150 pH benchtop meter) were recorded in the morning and afternoon. DO and pH were monitored to make sure that the environment was above minimum value requirements for the fish. For the first harvest, temperature was measured with the Sinergia Oxymeter at the same time as the DO and pH measurements, but all the other water temperature data

was recorded with temperature data loggers (Eclo Express Thermo) every 30 min. Flow rate (counting by filling a beaker) and photon irradiance (weather station) were also documented. The flow rate in the fish tanks and seaweed tanks were corrected daily. Photon irradiance data was measured every 10 min, and data between 6:00 and 20:00 was used.

2.2.2 Dissolved nutrients sampling and analysis

Ammonia levels in the fish tanks were aimed to be measured every morning at 9:00 using the Ammonium/Ammonia-Test from Tropic Marin following the instructions by the manufacturer, mainly to ensure appropriate levels for the fish. Ammonia in harvest 5 was only measured once due to circumstances.

Water samples for nutrient analysis were taken the day before the seaweed biomass measurements. Every week at 16:00, water samples were taken in duplicate from one fish tank and one seaweed tank per IRAS for the characterization of ammonia, nitrate, and nitrite. In week 3 and 5 water samples were taken in duplicate from the fish tanks to characterize the nutrient dynamics during the day. Every 2 hours samples were collected, with the first one at 6:00 and the last one at 22:00. This timeframe was chosen according to the study from Porter et al. (1987) that has shown that TAN excretion during night time is relatively stable. The water samples were taken with a syringe, filtered through a cellulose acetate membrane filter (0.45 µm pore size), collected in 20 mL acid washed plastic bottles, and immediately stored at -20°C to avoid alterations in nutrients by plankton growth, excretion, and decay before being sent to the laboratory for analysis. The data of the nutrient load were planned to be used to characterize and compare the dissolved nutrients in both systems. The data was not yet available to be presented in this work. For this reason, the Tropic Marin Ammonium/Ammonia-Test data was used instead for the nutrient analysis.

2.2.3 Microbial community sampling and analyses

The microbial community structure was used as proxy to evaluate water quality. Water samples were taken in each system to see how the fish density shapes the abundance of pico- and nanophytoplankton, bacterivores, and heterotrophic bacteria. Also, it was tested if the filtration units influenced the counts. Per week a total of six water samples were taken by filling a syringe with 20 mL of water sample when holding it ~10 cm under the

surface. The samples were collected in a 20 mL tinted glass flask, fixed with 160 µml glutaraldehyde (2.5% final concentration), and stored in the refrigerator for up to 7 days.

The composition and abundance of the phytoplankton were analyzed using epifluorescence microscopy (Haas, 1982). After 2 mL of the fixed sample was fluorescently stained with 40 µmL proflavine, it was filtered through a cellulose nitrate membrane filter (0.45 µm pore size, Ø25 mm) and Nuclepore track-edged membrane filter (0.20 µm pore size, Ø25 mm) using a vacuum filtration unit. The slides were prepared using non-fluorescent immersion oil (Cargile type A) and analyzed with a Leica DM LB epifluorescence microscope. Cyanobacteria (0.2 - 2 µm) were counted with green light, and pico- (0.2 - 2 µm) and nanophytoplankton (2 – 20 µm) with blue light at 787.5x total magnification for a total of 30 random visual fields. Heterotrophic bacteria (0 - 4 µm) were counted with blue light using a New Porton G12 ocular graticule. One square of the counting grid was seen as one field. Fields were counted to a minimum of 300 heterotrophic bacteria. It was assumed that the cells have a random distribution and with a counting precision of ±10% for bacteria and 20-30% for pico- and nanophytoplankton. A distinction in the microbial assemblage was made for the groups: cyanobacteria, pico-eukaryotes, diatoms, bacterivores, and heterotrophic bacteria. The abundance of the different groups was calculated with equation 1.

$$\textbf{Microbial abundance (cells L}^{-1}\textbf{)} = \frac{X * A * d}{a * n * V} \quad \textit{(equation 1)}$$

Where X = Total number of enumerated cells, A = Area of the polycarbonate membrane filter (mm^2), d = Correction factor for sample dilution by preservative, a = Area of fields observed (mm^2), n = Number of observed microscopic fields, and V = volume of filtered sample (L)

2.3 *Ulva* performance

2.3.1 Seaweed sampling & analyses

Once per week the *Ulva* was harvested and growth rate and biomass yield in FW and DW were calculated. Biomass yield is the amount of biomass growth in a certain amount of surface and time. All *Ulva* was taken out of the tank and water excess was removed by placing the seaweed on a mesh tray that was set inside in a 45° angle at room temperature. After 30 min, the tray was placed on a digital balance. Tissue (FW) was weighted once the tray stopped leaking water and the weight was stable while on the balance. After the measurements, excess biomass was removed, and *Ulva* was placed back at the original stocking density in a tank of randomized order to avoid positional influences in the

system. During the measurements, the water from the seaweed tanks was disposed, and the tanks were cleaned from debris and epiphytes and filled with clean water. Biomass subsamples were taken from excess biomass and put to dry at 60°C for 3≥ days in the oven. To convert the data from FW to DW, a linear regression equation was calculated to find the relationship between *Ulva* FW and DW. After measuring the tissue (DW), subsamples from each density treatment were used for the determination of carbon and nitrogen (C-N) content (%) to then calculate the N removal by seaweed biomass in the system. The tissue was ground to a fine powder with a pestle and mortar. Ground samples were kept in a dissector inside an Eppendorf until analysis. T0 (N content of *Ulva* before implemented in the IRAS) and samples from each stocking density at the end of Cycle 1 and of Cycle 2 were sent for C-N analysis at an external laboratory. Data collected was used to calculate *Ulva* specific growth rate (% day^{-1}), biomass yield (g DW m^{-2} d^{-1}), and N yield (g N m^{-2} d^{-1}). After harvesting, the reproductive state of the seaweed was also assessed. A random sample of thalli was taken from each replicate of stocking density treatment 2. The pigment distribution of the tissue was studied by eye and microscope (Nikon Eclipse Ci; 40x magnification), and microscopic photos were taken with NIS-Elements imaging software.

2.3.2 Growth and yield estimation

The SGR (% d^{-1}) was calculated with the produced biomass (g FW) of the seaweed with equation 2, the most accurate formula suggested by Yong et al. (2013). The calculated linear regression equation was used to convert total biomass yield per tank in FW to DW. The biomass yield (g m^{-2} d^{-1}) was calculated with equation 3 for both FW and DW. N removal by *Ulva* biomass production was calculated by multiplying the biomass yield (g DW) by the nitrogen content of *Ulva* tissue with equation 4.

$$\textbf{Daily SGR (\% d}^{-1}\textbf{)} = [(W_t / W_0)^{1/t} - 1] \times 100 \quad \textit{(equation 2)}$$

$$\textbf{Daily Y (g m}^{-2}\ \textbf{d}^{-1}\textbf{)} = [(W_t - W_0) / A] / t \quad \textit{(equation 3)}$$

$$\textbf{N yield (g N)} = Y \times Nc \quad \textit{(equation 4)}$$

W_0 = initial biomass (g), W_t = final biomass (g), t = total of culture days, A = area of surface cultivation tank (m^2), Y = biomass yield (g DW), Nc = nitrogen content (%).

2.4 Statistical analyses

During the experiment the fish densities were reduced due to high ammonia levels in the fish tanks, so for this reason the data before and after the fish density change were analyzed separately and referred to as Cycle 1 and 2 in the Results.

The results were statistically analyzed using R software package version 4.1.2. Data normality and homogeneity were tested beforehand using the Shapiro-Wilk and Levene's test. When assumptions were not met, non-parametric tests were used to compare medians of the data. The Two-way ANOVA was used to check for potential interaction effect between the fish density (IRAS High-F and Low-F) and stocking density treatments (1, 2, and 3), and averages were compared with the Tukey HSD test. SGR and yield by *Ulva* in the two experimental IRAS were compared with the Mann-Whitney test. N content was compared at different time intervals with the independent T-test. The Kruskal-Wallis test was used to compare medians of the microbial abundances between functional units in the IRAS, and the microbial groups between the systems were compared using the Mann-Whitney test. Climatological and physical-chemical variables were averaged over a period of 7 days prior to sampling. DO and pH were compared using the Mann-Whitney test.

Data was given as mean (*M*) ± standard deviation (*SD*), and in median (*Md*) for non-parametric tests. The null hypothesis was rejected at a significance level (α) of 5%.

3. Results

The results of the water quality in two IRAS were compared for two different cycles. *Ulva* harvests 1 and 2 belong to Cycle 1 where IRAS High-F and Low-F held 20 kg and 10 kg fish m^{-3}, respectively. *Ulva* harvests 3, 4 and 5 belong to Cycle 2 where the fish densities were reduced to 10 and 5 kg fish m^{-3}. *Ulva* stocking densities 1, 2, and 3 kg FW m^{-2} are referred to as density treatments 1, 2, and 3, respectively. Every week *Ulva* was measured in growth rate and biomass yield. In addition, biofiltration, water quality parameters, and microbial community composition of the IRAS were assessed.

3.1 Water quality

3.1.1 General environmental conditions

The environmental conditions in the seaweed tanks per cycle are given in Table 3.1. Average ammonia level in IRAS High-F was 1.26 mg L^{-1} in Cycle 1 and 0.43 mg L^{-1} in Cycle 2. For IRAS Low-F this was 0.23 mg L^{-1} in Cycle 1 and 0.07 mg L^{-1} in Cycle 2. Water temperature and photon irradiation in Cycle 2 were generally higher than in Cycle 1. In Cycle 1 water in the fish tanks were purged two times per day and in equal water renewal quantity for both IRAS because of the high ammonia levels in IRAS High-F. There was an evident difference in the accumulation of solids on the bioballs that developed during the experiment as well (Figure 3.1).

Table 3.1: Environmental growth conditions for each Ulva harvest for Cycle 1 and 2. Data represents means ± SD.

	***Ulva* Harvest**	**Water temperature (°C)**	**Photon irradiation (W m^{-2})**	**Flow rate (L h^{-1})**
Cycle 1	1	*17.7 ± 1.7*	258.7 ± *193.9*	624.9 ± *103.4*
	2	*21.7 ± 1.9*	317.0 ± *200.0*	514.0 ± *0.0*
	Average	***19.3 ± 2.4***	***287.8 ± 199.0***	***569.5 ± 91.7***
Cycle 2	3	*23.5 ± 2.1*	340.1 ± *197.0*	479.5 ± *32.1*
	4	*24.0 ± 1.5*	325.3 ± *197.6*	450.0 ± *0.0*
	5	*23.7 ± 1.7*	290.1 ± *187.4*	450.0 ± *0.0*
	Average	***23.7 ± 1.8***	***320.0 ± 195.3***	***459.8 ± 29.6***

Figure 3.1: Accumulation of organic matter on bioballs in IRAS High-F (left) and Low-F (right) during the experiment.

3.1.2 Microbial community

3.1.2.1 Microbic observations during experiment

During the microscopic observations, the phytoplankton groups were divided into Cyanobacteria, picoeukaryotes, and diatoms. Bacterivores included dinoflagellates, nanoflagellates, ameboids and cryptophytes. Microscopic views of the different phytoplankton groups are shown in Figure 3.2. The Kruskal-Wallis test showed no significant differences in any microbial groups between the three locations (Fish, Seaweeds, and Filter unit), so the abundances of the three samples were averaged for each harvest.

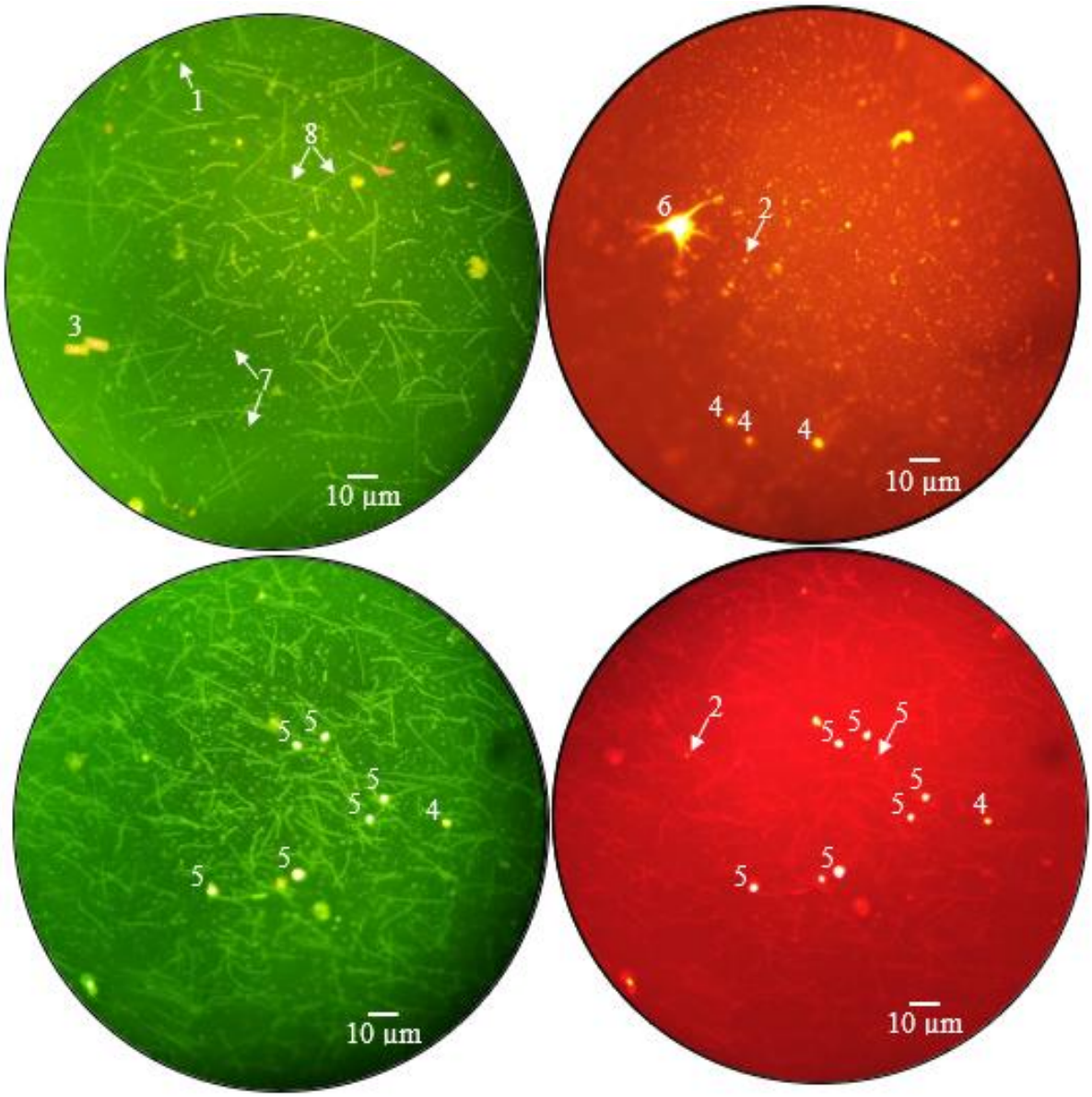

Figure 3.2: Fluorescence micrographs under blue (green background) and green (red background) light of picoeukaryotes (1), cyanobacteria (2), diatoms (3), nanoflagellates (4), cryptophytes (5), ameboid (6), heterotrophic bacteria (7), and chain-forming bacteria (8). Bottom two images are the same photo taken under different light.

3.1.2.2 Microbes, temperature, and ammonia during experiment

The microbial community structure during the experiment varied among the *Ulva* harvests. The phytoplankton abundances (cyanobacteria, picoeukaryotes, diatoms) increased from harvest 1 to 2, but crashed in harvest 3 after a large water exchange took place to reduce the fish densities in both IRAS for Cycle 2. The abundances recovered in the harvests that followed. The average temperature was higher in Cycle 2 compared with Cycle 1 (Figure 3.3). The heterotrophic abundances seem to follow the ammonia levels (Figure 3.4), and bacterivores seem to follow heterotrophic bacteria abundance (Figure 3.5). For all the microbial groups, the highest abundances were observed in IRAS High-F, except for harvest 1.

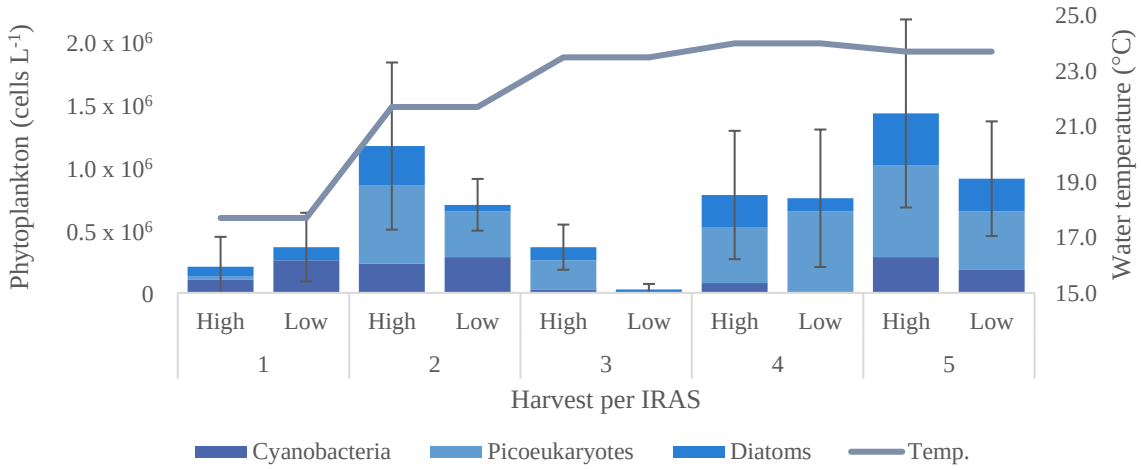

Figure 3.3: Abundance of the phytoplankton groups cyanobacteria, picoeukaryotes, and diatoms for IRAS High-F and Low-F the day before Ulva harvest and average temperature. Data expressed as means ± SD (n = 3).

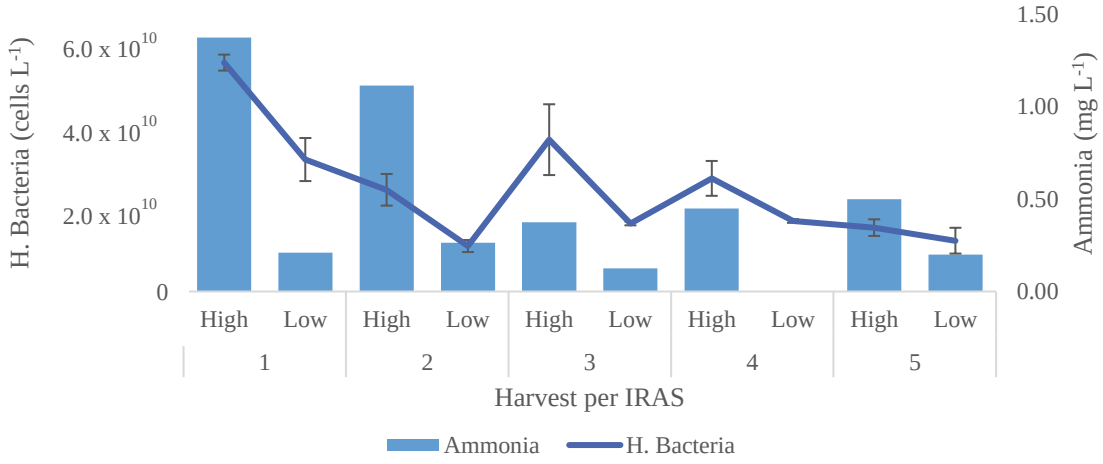

Figure 3.4: Abundance of heterotrophic bacteria for IRAS High-F and Low-F the day before Ulva harvest and ammonia level. Data expressed as means ± SD (n = 3).

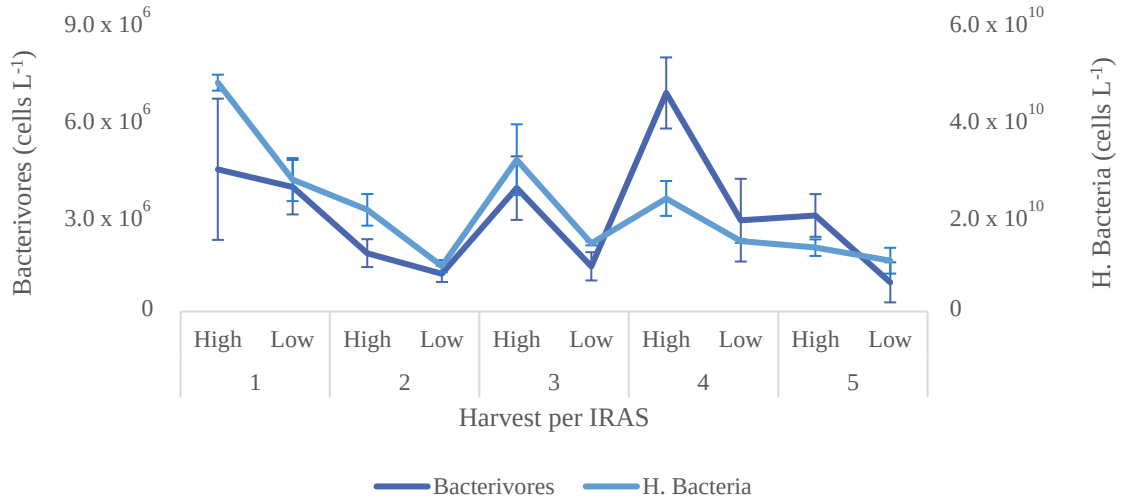

Figure 3.5: Abundance of heterotrophic bacteria and bacterivores for IRAS High-F and Low-F the day before Ulva harvest. Data expressed as means ± SD (n = 3).

3.1.2.3 Microbial community structure in different IRAS

The microbial community abundances (cells L^{-1}) were compared between the two IRAS to assess a difference in community structure (Table 3.2). The medians of the groups were used in the statistical tests as assumptions were violated by multiple microbial groups.

In Cycle 1, the Mann Whitney test showed no significant differences of any microbial group between the two IRAS. In Cycle 2, the Mann Whitney test revealed that heterotrophic bacteria were significantly higher in IRAS High-F (Md = 2.27 x 10^{10}, n = 9) compared to IRAS Low-F (Md = 1.46 x 10^{10}, n = 9), z = 2.297, p = 0.01). Also, bacterivores were significantly higher in IRAS High-F (Md = 3.76 x 10^6, n = 9) compared to IRAS Low-F (Md = 1.65 x 10^6, n = 9), z = 3.046, $p < 0.01$). In average there were 74% more heterotrophic bacteria observed in IRAS High-F (2.37 x 10^{10} ± 9.34 x 10^9) than in Low-F (1.37 x 10^{10} ± 2.47 x 10^9) and 164% more bacterivores observed in IRAS High-F (4.74 x 10^6 ± 2.00 x 10^6) than in Low-F (1.79 x 10^6 ± 1.20 x 10^6; Figure 3.6 & Figure 3.7). There was no significant difference found between the IRAS in the other microbial groups.

Table 3.2: Microbial abundance (cells L^{-1}) in IRAS High-F and Low-F in Cycle 1 (n = 6) and 2 (n = 9). H : L is ratio of IRAS High-F : Low-F. Data represents means ± SD.

	Cycle 1			Cycle 2		
IRAS	**High-F**	**Low-F**	**H : L**	**High-F**	**Low-F**	**H : L**
Cyanobacteria	$1.99 \times 10^5 \pm 2.13 \times 10^5$	$3.21 \times 10^5 \pm 2.51 \times 10^5$	62%	$1.53 \times 10^5 \pm 2.38 \times 10^5$	$7.14 \times 10^4 \pm 1.28 \times 10^5$	214%
Picoeukaryotes	$3.82 \times 10^5 \pm 4.62 \times 10^5$	$2.14 \times 10^5 \pm 4.05 \times 10^5$	179%	$5.51 \times 10^5 \pm 4.10 \times 10^5$	$4.38 \times 10^5 \pm 5.13 \times 10^5$	126%
Diatoms	$2.29 \times 10^5 \pm 2.38 \times 10^5$	$9.18 \times 10^4 \pm 8.21 \times 10^4$	250%	$3.06 \times 10^5 \pm 2.97 \times 10^5$	$1.53 \times 10^5 \pm 2.05 \times 10^5$	200%
Bacterivores	$3.26 \times 10^6 \pm 2.10 \times 10^6$	$2.63 \times 10^6 \pm 1.65 \times 10^6$	124%	$4.74 \times 10^6 \pm 2.00 \times 10^6$	$1.79 \times 10^6 \pm 1.20 \times 10^6$	264%
H. bacteria	$3.58 \times 10^{10} \pm 1.53 \times 10^{10}$	$1.92 \times 10^{10} \pm 1.07 \times 10^{10}$	186%	$2.37 \times 10^{10} \pm 9.34 \times 10^9$	$1.37 \times 10^{10} \pm 2.47 \times 10^9$	174%

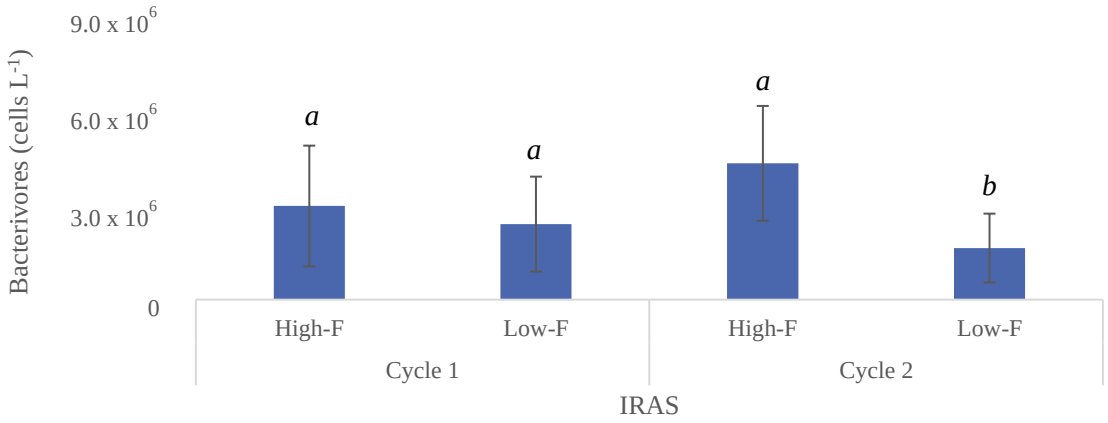

Figure 3.6: Abundance of bacterivores in IRAS High-F and Low-F in Cycle 1 (n = 6) and 2 (n = 9). Columns sharing a letter indicate no significant difference in performance in IRAS High-F and in Low-F, calculated at $p < 0.05$. Data represents mean ± SD.

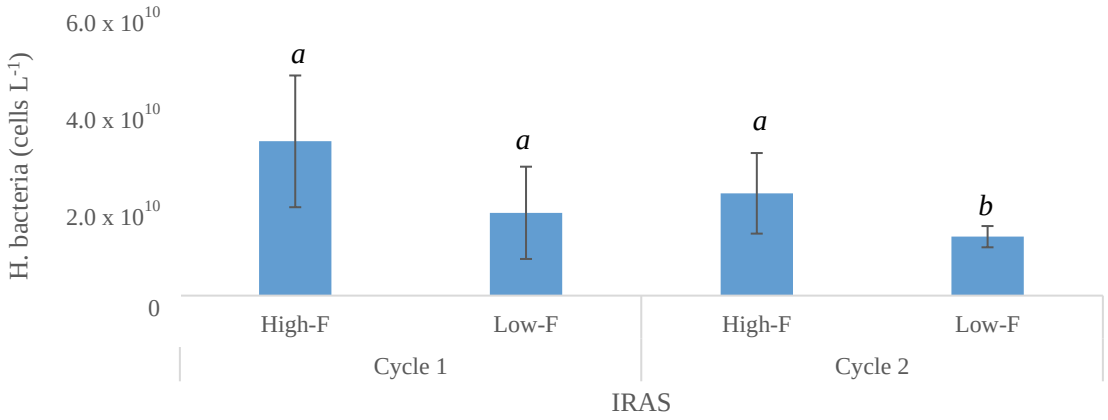

Figure 3.7: Abundance of heterotrophic bacteria in IRAS High-F and Low-F in Cycle 1 (n = 6) and 2 (n = 9). Columns sharing a letter indicate no significant difference in performance in IRAS High-F and in Low-F, calculated at $p < 0.05$. Data represents mean ± SD.

3.2 *Ulva* performance

3.2.1 Fresh to dry weight conversion

Simple linear regression was used to test if g FW significantly predicted g DW. The fitted regression model was: y = 0.157x – 0.2812. The overall regression was statistically significant (R^2 = 0.732, *F(1, 88)* = 240.5, $p < 0.01$). As expected, it was found that g FW significantly predicted g DW (β = 0.157, $p < 0.01$; Figure 3.8), and therefore the equation was used to estimate DW.

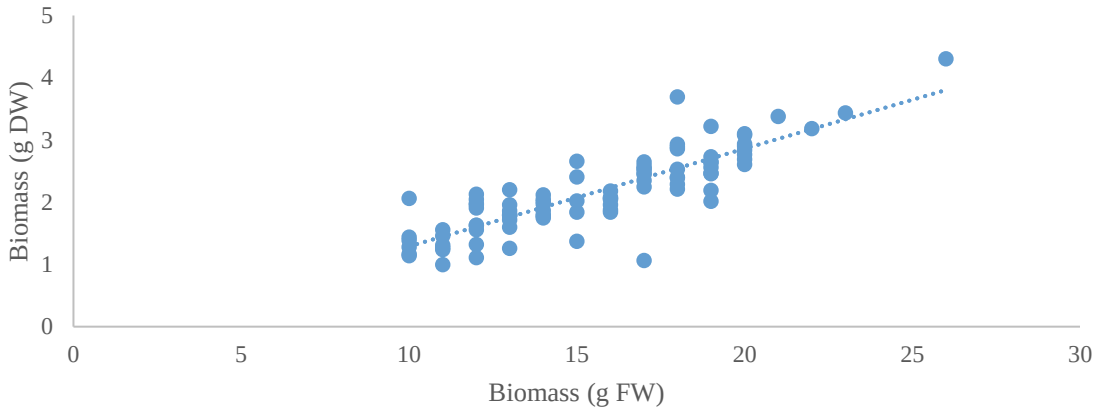

Figure 3.8: The relationship between g FW and g DW of Ulva sp., data from all harvests. Regression line: y = 0.157x – 0.2812, R^2 = 0.732, p < 0.01)

3.2.2 Growth and yield at different fish densities

It was hypothesized that IRAS High-F would show higher *Ulva* performance than Low-F, but the data did not support this (Table 3.3). Non-parametric tests were used as assumptions were not met.

In Cycle 1, there was no significant difference found in SGR (% d^{-1}) and yield (g FW m^{-2} d^{-1}) between the IRAS. In Cycle 2, the Mann-Whitney test revealed that SGR was significantly lower in IRAS High-F (*Md* = 19.0, *n* = 27) compared to IRAS Low-F (*Md* = 24.4, *n* = 27), *z* = -2.534, *p* = 0.01. The yield (FW) was also significantly lower in IRAS High-F (*Md* = 825.0, *n* = 27) compared to IRAS Low-F (*Md* = 1046.3, *n* = 27), *z* = -3.910, $p < 0.01$ (Figure 3.10 & Figure 3.9).

Table 3.3: Daily SGR and yield of Ulva sp. for IRAS High-F and Low-F for Cycle 1 (n = 18) and Cycle 2 (n = 27). Data represents means ± SD.

	IRAS	**SGR (% d^{-1})**	**Yield (g FW m^{-2} d^{-1})**	**Yield (g DW m^{-2} d^{-1})**
Cycle 1	High-F	*22.4 ± 7.3*	*760.4 ± 261.1*	*119.2 ± 41.0*
	Low-F	*23.2 ± 8.1*	*824.5 ± 297.3*	*129.3 ± 46.7*
Cycle 2	High-F	*21.4 ± 6.1*	*770.5 ± 259.9*	*120.8 ± 40.8*
	Low-F	*26.7 ± 8.7*	*1064.8 ± 227.2*	*167.0 ± 35.7*

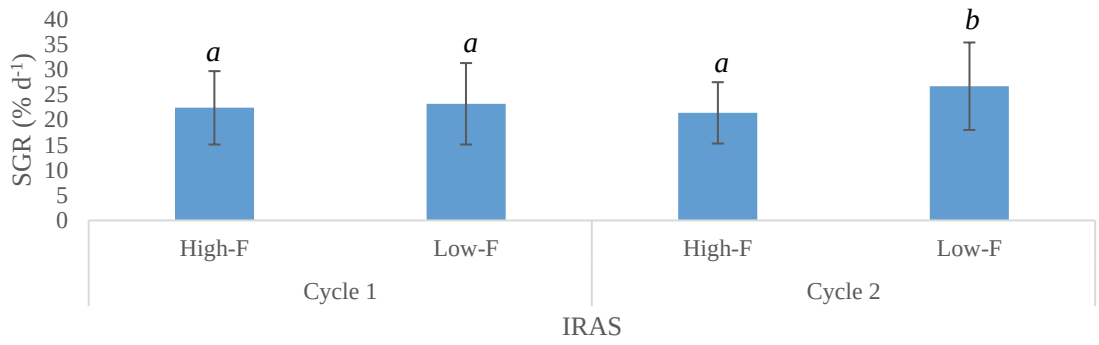

Figure 3.10: SGR of Ulva sp. in IRAS High-F and Low-F in Cycle 1 (n = 18) and Cycle 2 (n = 27). Data represents means ± SD. Columns sharing a letter indicate no significant difference in performance between the IRAS within that cycle, calculated at p < 0.05.

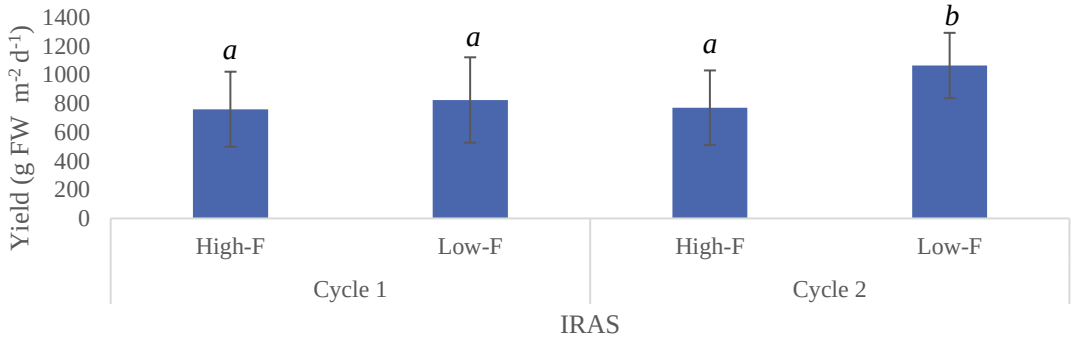

Figure 3.9: Yield of Ulva sp. in IRAS High-F and Low-F in Cycle 1 (n = 18) and Cycle 2 (n = 27). Data represents mean ± SD. Columns sharing a letter indicate no significant difference in performance between the IRAS within that cycle, calculated at p < 0.05

3.2.3 Growth and yield at different *Ulva* stocking densities

Cycle 1

For Cycle 1, a Two-way ANOVA was performed to analyze the effect of fish density and *Ulva* stocking density on SGR. The test revealed that there was no statistically significant interaction between the effects of fish density and *Ulva* stocking density, $F(2, 30) = 0.090$, $p = 0.92$. Simple main effects analyses showed that *Ulva* stocking density did have a statistically significant effect on SGR ($p < 0.01$), but fish density did not ($p = 0.57$). Tukey´s HSD test for multiple comparisons showed that the mean SGR of *Ulva* density 1 (31.2% d^{-1} ± 5.8) was significantly higher than *Ulva* densities 2 (20.9% d^{-1} ± 4.5; $p < 0.01$) and 3 (16.3% d^{-1} ± 2.2; $p < 0.01$).

For yield (FW) the Two-way ANOVA test revealed that there was no statistically significant interaction between the effects of fish density and *Ulva* stocking density, $F(2, 30) = 0.01$, $p = 0.99$. Simple main effects analyses showed that *Ulva* stocking density had no statistically significant effect on yield ($p = 0.98$), and the same applied for fish density ($p = 0.52$; Table 3.4 & Figure 3.11).

Table 3.4: Ulva performance in IRAS for stocking densities 1, 2, and 3 kg m^{-2} in Cycle 1. Data represents mean ± SD (n = 12).

Ulva stocking density	**SGR (% d^{-1})**	**Yield (g FW m^{-2} d^{-1})**	**Yield (g DW m^{-2} d^{-1})**
1	*31.2 ± 5.8*	*805.0 ± 356.4*	*126.2 ± 56.0*
2	*20.9 ± 4.5*	*789.6 ± 298.7*	*123.8 ± 46.9*
3	*16.3 ± 2.2*	*782.8 ± 169.8*	*122.7 ± 26.7*

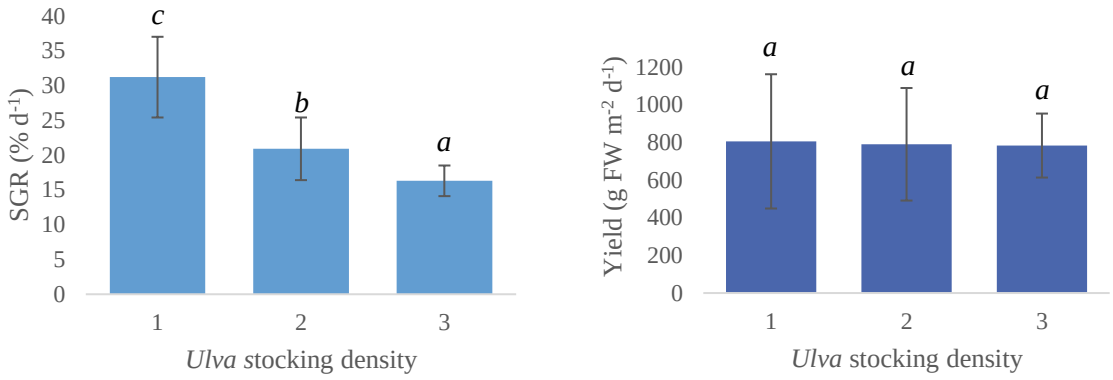

Figure 3.11: SGR (left) and yield (right) for the Ulva stocking densities 1, 2, and 3 kg m^{-2} for Cycle 1. Data represents means ± SD. Columns sharing a letter indicate no significant difference in performance, calculated at $p < 0.05$.

Cycle 2

For Cycle 2, a Two-way ANOVA was performed to analyze the effect of fish density and *Ulva* stocking density on SGR. The test revealed that there was a statistically significant interaction between the effects of fish density and *Ulva* stocking density, $F(2, 48) = 9.141$, $p < 0.01$. The main effects cannot be examined separately as the true response for SGR for *Ulva* stocking density depends on fish density, so the values were separated for each IRAS (Table 3.5). In IRAS High-F, One-way ANOVA showed that SGR for stocking density 1 (25.5 ± 7.5) was significantly higher than 3(16.5 ± 1.2), $F(2, 24) = 7.694$, $p < 0.01$. On the other hand, yield (FW) in IRAS High-F was for stocking density 1 (*603.7 ± 299.6)* significantly lower than for stocking density 2, (891.0 ± *269.4*), $F(2, 24) = 3.541$, $p = 0.04$.

Similar results were found in IRAS Low-F. The One-way ANOVA showed that SGR was significantly higher for stocking density 1 (*37.6 ± 3.7)* than stocking density 2 (*24.2 ± 2.7*) and 3 (*18.3 ± 2.3*), $F(2, 24) = 98.31$, $p < 0.01$. There was no significant difference found for yield (FW) between the *Ulva* stocking densities, $F(2, 24) = 3.114$, $p = 0.06$ (Figure 3.12 & Figure 3.13).

Table 3.5: SGR and yield for Ulva stocking density 1, 2, and 3 kg m^{-2} for IRAS High-F and Low-F in Cycle 2. Data represents means ± SD (n = 9).

***Ulva* stocking density**	**IRAS**	**SGR (% d^{-1})**	**Yield (g FW m^{-2} d^{-1})**	**Yield (g DW m^{-2} d^{-1})**
1	High-F	25.5± 7.5	603.7 ± 299.6	94.6 ± 47.0
	Low-F	37.6 ± 3.7	1,203.5 ± 244.0	188.8 ± 38.3
Average		**31.5 ± 8.4**	**903.6 ± 406.8**	**141.7 ± 93.9**
2	High-F	22.1 ± 4.0	891.0 ± 269.4	139.7 ± 42.3
	Low-F	24.2 ± 2.7	1,026.1 ± 192.7	160.9 ± 30.3
Average		**23.1 ± 3.5**	**958.5 ± 237.6**	**150.3 ± 37.3**
3	High-F	16.5 ± 1.2	816.8 ± 85.4	128.1 ± 13.4
	Low-F	18.3 ± 2.3	964.9 ± 191.1	151.3 ± 30.0
Average		**17.4 ± 2.0**	**917.7 ± 162.6**	**139.7 ± 25.5**

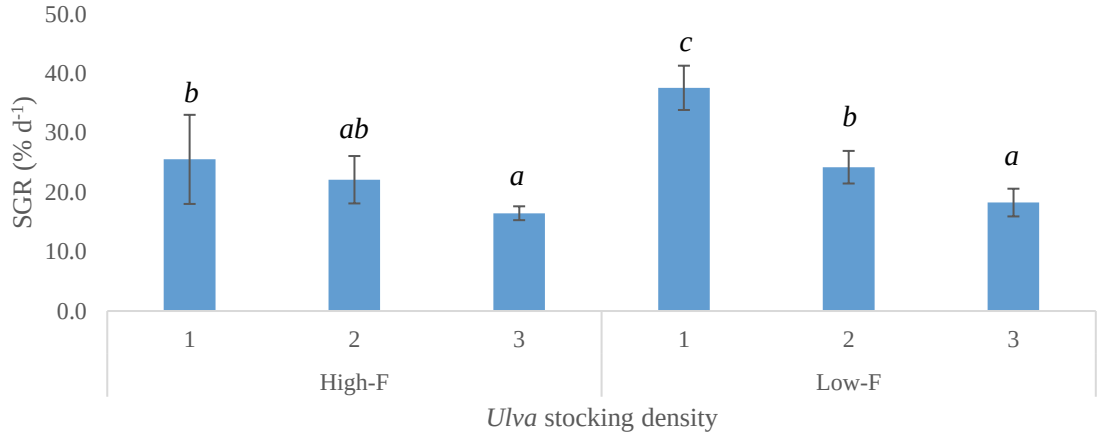

Figure 3.12: SGR of stocking densities 1, 2, and 3 kg m^{-2} in IRAS High-F and Low-F in Cycle 2. Columns sharing a letter indicate no significant difference in performance in IRAS High-F and in Low-F, calculated at $p < 0.05$. Data represents mean ± SD (n = 9).

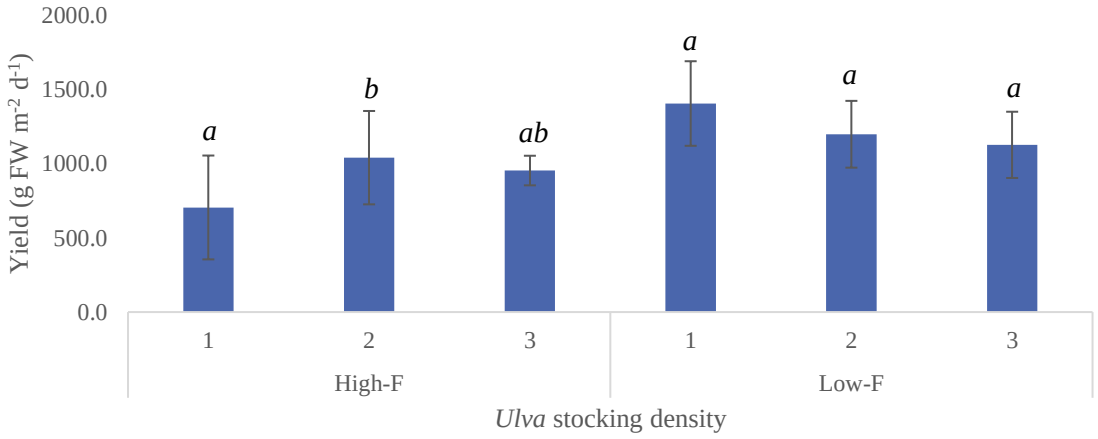

Figure 3.13: Yield of Ulva stocking densities 1, 2, and 3 kg m^{-2} for IRAS High-F and Low-F in Cycle 2. Columns sharing a letter indicate no significant difference in performance in IRAS High-F and in Low-F, calculated at $p < 0.05$. Data represents mean ± SD (n = 9).

3.2.4 Growth and yield in different harvests

SGR (% d^{-1}) and yield (g FW m^{-2} d^{-1}) for each harvest in both IRAS of *Ulva* were compared between the harvests (Figure 3.14 & Figure 3.15). One-way ANOVA test showed no significant difference in SGR among the harvests for both IRAS High-F, $F(4, 40) = 2.549$, $p > 0.05$, and IRAS Low-F, $F(4, 40) = 1.616$, $p = 0.19$. The highest SGR however was observed in harvest 5 for both IRAS High-F ($M = 25.3 \pm 7.6$) and Low-F ($M = 28.1 \pm 9.2$). One-way ANOVA test did show a significant difference in yield among the harvests for both IRAS High-F, $F(4, 40) = 6.599$, $p < 0.01$, and IRAS Low-F, $F(4, 40) = 15.430$ $p < 0.01$. Tukey´s HSD test showed that for IRAS High-F harvest 5 ($M = 966.5 \pm 183.7$) was significantly higher in yield than harvest 1 ($M = 609.4 \pm 100.0$; $p = 0.01$) and 3 ($M = 559.6 \pm 207.8$; $p < 0.01$). For IRAS Low-F harvest 5 ($M = 1159.9 \pm 196.1$) was only significantly higher than harvest 1 ($M = 561.4$; $p < 0.01$). The fish density reduction happened after harvest 2 and for both SGR and yield a reduction in performance was measured in harvest 3. Per cycle performance appears to increase over time.

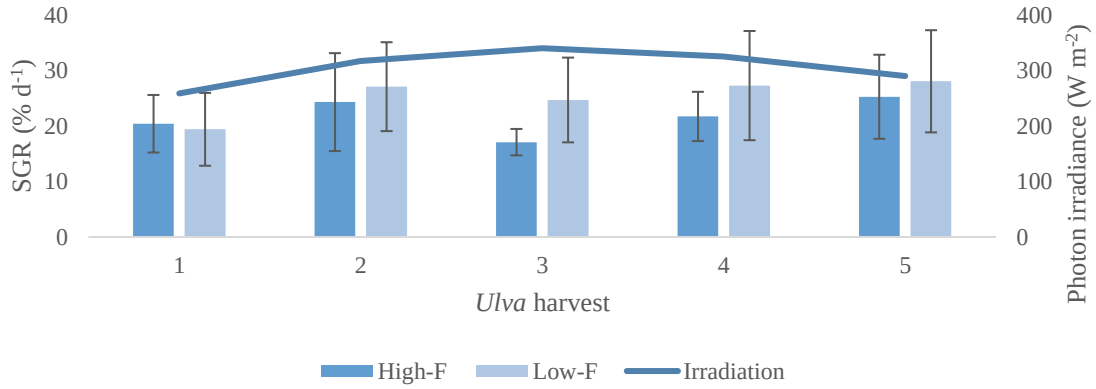

Figure 3.14: SGR (% d^{-1}) by Ulva sp. in IRAS High-F and Low-F per harvest. Cycle 1 includes Ulva harvest 1 and 2, and Cycle 2 includes harvest 3 t/m 5. Data expressed as mean ± SD (n = 9).

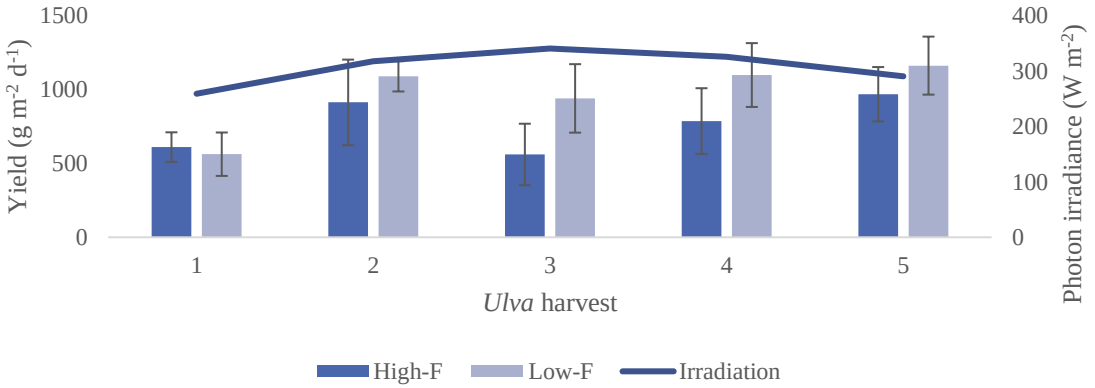

Figure 3.15: Yield (g FW m^{-2} d^{-1}) by Ulva sp. in IRAS High-F and Low-F per harvest. Cycle 1 includes Ulva harvest 1 and 2, and Cycle 2 includes harvest 3 t/m 5. Data expressed as means ± SD (n = 9).

3.2.5 *Ulva* N removal

Independent T-test showed that *Ulva* in IRAS High-F at the end of Cycle 1 (M = 3.82 ± 0.15) was significantly higher in N content than at time of stocking (T0; M = 1.30 ± 0.03), $t\ (9.637)$ = 48.000, p < 0.01. In IRAS Low-F N content was also higher in Cycle 1 (M = 2.99 ± 0.11) than at T0 (M = 1.30 ± 0.03), $t\ (10.000)$ = 41.698, p < 0.01. Independent T-test showed that *Ulva* in IRAS High-F at the end of Cycle 1 (M = 3.82 ± 0.15) was significantly higher in N content than at the end of Cycle 2 (M = 2.94 ± 0.16), $t\ (15.943)$ = 12.319, p < 0.01. In IRAS Low-F N content was also higher in Cycle 1 (M = 2.99 ± 0.11) than in Cycle 2 (M = 2.56 ± 0.30), $t\ (10.024)$ = 4.034, p < 0.01.

N content in *Ulva* biomass increased in both IRAS compared with the N yield of the T0 measurements. N yield decreased when ammonia level decreased (Figure 3.16 & Table 3.6). The highest average N yield (4.55 g N m^{-2} d^{-1}) was seen at 1.26 mg L^{-1} TAN in Cycle 1, followed by 4.28 g N m^{-2} d^{-1} at 0.07 mg L^{-1} TAN in Cycle 2. Although ammonia level and N content was at its lowest in Cycle 2 in IRAS Low-F, biomass yield (g DW) was much higher than in the previous cycle, resulting in a higher N yield than in IRAS High-F.

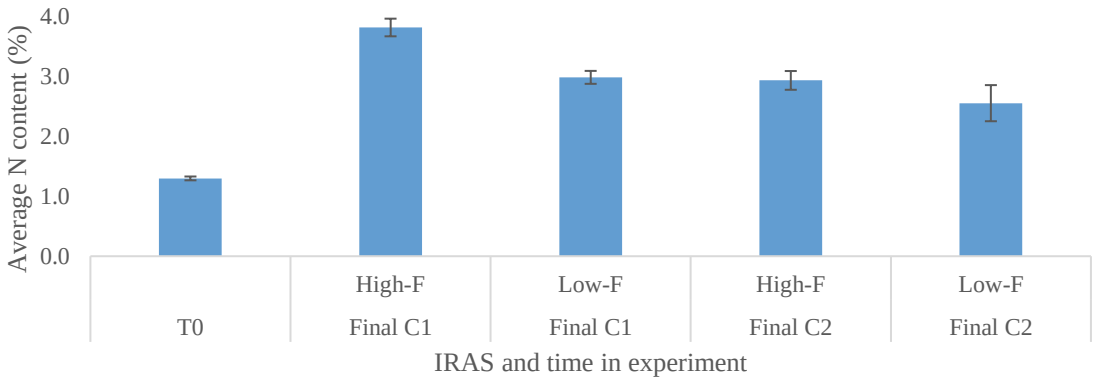

Figure 3.16: Initial (T0; n = 3), End of Cycle 1 (C1; n = 9) and End of Cycle 2 (C2; n = 9) N content (%) in Ulva biomass in IRAS High-F and Low-F. Data represents mean ± SD of N content.

Table 3.6: Ammonia and N content of Ulva biomass at T0, at the end of Cycle 1 and at the end of Cycle 2. Data represents mean ± SD.

	IRAS	Ammonia (mg L^{-1})	Biomass yield (g DW m^{-2} d^{-1})	N content (%)	N yield (g N m^{-2} d^{-1})
T0				*1.30 ± 0.03*	
Cycle 1	High-F	1.26 ± 0.86	119.21 ± 41.00	3.82 ± 0.15	4.55 ± 1.57
	Low-F	0.23 ± 0.18	129.27 ± 46.69	2.99 ± 0.11	3.87 ± 1.40
Cycle 2	High-F	0.43 ± 0.12	120.80 ± 40.81	2.94 ± 0.16	3.55 ± 1.20
	Low-F	0.07 ± 0.11	167.01 ± 35.67	2.56 ± 0.30	4.28 ± 0.91

3.2.6 *Ulva* cultivation and environment

Ulva performance parameters per harvest were compared to see the effects of seaweed cultivation on the water quality parameters DO (mg L^{-1}) and pH. The data does not suggest that *Ulva* performance parameters significantly influence DO and pH in this system as they are generally stable, while performance variates over the harvests. Nevertheless, there was a general decline in DO and rise in pH during the day in the seaweed tanks (Table 3.7). An unknown but assumed to be fixed amount of pure oxygen was continuously added in both systems, so fluctuations in DO were assumed to be caused by biological activities.

In Cycle 1, the Mann Whitney test revealed that DO was significantly higher in IRAS High-F (*Md* = 9.4, *n* = 75) compared to IRAS Low-F (*Md* = 9.1, *n* = 75), *z* = 2.440, *p* < 0.01). The pH was significantly lower in IRAS High-F (*Md* = 8.1, *n* = 72) compared to IRAS Low-F (*Md* = 8.2, *n* = 72), *z* = -2.840, *p* < 0.01). There was no significant difference found in DO between morning and afternoon in both IRAS High-F (*z* = -0.520, *p* = 0.60) and Low-F (*z* = -1.290, *p* = 0.20). However, the pH was significantly higher in IRAS Low-F from the afternoon (*Md* = 8.2, *n* = 33) compared to the morning (*Md* = 8.0, *n* = 39), *z* = 3.654, *p* < 0.01. Similarly, IRAS Low-F was higher in pH in the afternoon (*Md* = 8.4, *n* = 33) than the morning (*Md* = 8.0, *n* = 39), *z* = 5.176, *p* < 0.01. The averages are shown in Table 3.8.

In Cycle 2, the Mann Whitney test also showed no significant difference in DO between the two IRAS, but the pH was significantly lower in IRAS High-F (*Md* = 8.0, *n* = 117) compared to IRAS Low-F (*Md* = 8.2, *n* = 117), *z* = -6.610, *p* < 0.01. During the day, DO in IRAS High-F was significantly lower in the afternoon (*Md* = 9.1, *n* = 54) compared to the morning (*Md* = 9.4, *n* = 61), *z* = -2.077, *p* = 0.02. DO in IRAS Low-F was also significantly lower in the afternoon (*Md* = 9.0, *n* = 54) than in the morning (*Md* = 9.4, *n* = 63), *z* = -4.002, *p* < 0.01. The pH in IRAS High-F was significantly higher in the afternoon (*Md* = 8.2, *n* = 54) than in the morning (*Md* = 7.8, *n* = 63), *z* = 9.023, *p* < 0.01. The pH in IRAS Low-F was also significantly higher from the afternoon (*Md* = 8.5, *n* = 54) compared to the morning (*Md* = 8.0, *n* = 63), *z* = 9.184, *p* < 0.01. The averages are shown in Table 3.8.

Table 3.7: DO and pH in the morning and afternoon in the seaweed tanks for IRAS High-F and Low-F in Cycle 1 and 2. Data represents mean ± SD of daily measurements.

	Cycle 1		Cycle 2	
	Morning	**Afternoon**	**Morning**	**Afternoon**
DO (mg L^{-1})				
High-F	*9.7 ± 1.1*	*9.6 ± 1.0*	*9.4 ± 0.7*	*9.2 ± 0.6*
Low-F	*9.3 ± 0.7*	*9.1 ± 0.8*	*9.5 ± 0.6*	*9.1 ± 0.4*
pH				
High-F	*8.0 ± 0.2*	*8.2 ± 0.2*	*7.8 ± 0.1*	*8.2 ± 0.1*
Low-F	*8.0 ± 0.3*	*8.3 ± 0.2*	*8.0 ± 0.1*	*8.5 ± 0.1*

Table 3.8: Weekly water quality parameters in the seaweed tanks and Ulva performance per harvest in IRAS High-F and Low-F. Cycle 1 includes Ulva harvest 1 and 2, and Cycle 2 includes harvest 3 t/m 5. Data represents mean ± SD.

Cycle	Harvest	DO (mg O_2 L^{-1})	pH	SGR (% d^{-1})	Yield (g FW m^{-2} d^{-1})	Yield (g DW m^{-2} d^{-1})
High-F						
1	1	*10.2 ± 1.2*	*8.0 ± 0.2*	*20.4 ± 5.2*	*609.3 ± 100.0*	*95.5 ± 15.7*
	2	*9.2 ± 0.5*	*8.1 ± 0.2*	*24.3 ± 8.8*	*911.4 ± 289.0*	*142.9 ± 45.4*
	Average	***9.7 ± 1.0***	***8.1 ± 0.2***	***22.4 ± 7.3***	***760.4 ± 261.1***	***119.2 ± 41.0***
2	3	*9.5 ± 0.6*	*8.0 ± 0.2*	*17.1 ± 2.4*	*559.6 ± 207.7*	*87.7 ± 32.6*
	4	*9.2 ± 0.5*	*8.0 ± 0.3*	*21.7 ± 4.4*	*785.3 ± 221.9*	*123.1 ± 34.8*
	5	*9.4 ± 0.8*	*8.0 ± 0.2*	*25.3 ± 7.6*	*966.4 ± 183.7*	*151.6 ± 28.8*
	Average	***9.3 ± 0.6***	***8.0 ± 0.2***	***21.4 ± 6.1***	***770.4 ± 260.0***	***120.8 ± 40.8***
Low-F						
1	1	*9.6 ± 0.8*	*8.0 ± 0.4*	*19.4 ± 6.6*	*561.3 ± 146.7*	*87.9 ± 23.0*
	2	*8.9 ± 0.5*	*8.3 ± 0.2*	*27.1 ± 8.0*	*1087.6 ± 102.4*	*170.6 ± 16.1*
	Average	***9.2 ± 0.7***	***8.2 ± 0.3***	***23.2 ± 8.1***	***824.5 ± 297.3***	***129.3 ± 46.7***
2	3	*9.3 ± 0.5*	*8.3 ± 0.2*	*24.7 ± 7.6*	*938.3 ± 231.3*	*147.1 ± 36.3*
	4	*9.1 ± 0.4*	*8.2 ± 0.3*	*27.3 ± 9.8*	*1096.2 ± 215.6*	*171.9 ± 33.9*
	5	*9.4 ± 0.7*	*8.3 ± 0.3*	*28.1 ± 9.2*	*1159.8 ± 196.0*	*181.9 ± 30.8*
	Average	***9.3 ± 0.5***	***8.3 ± 0.3***	***26.7 ± 8.7***	***1064.8 ± 227.2***	***167.0 ± 35.7***

3.2.7 Reproductive state

Reproductive state of *Ulva* was assessed. During the experiment reproduction state was observed in IRAS High-F for harvest 1, and in Low-F for harvest 2 and 5. All the other samples were in vegetative state. See Figure 3.17 and Figure 3.18.

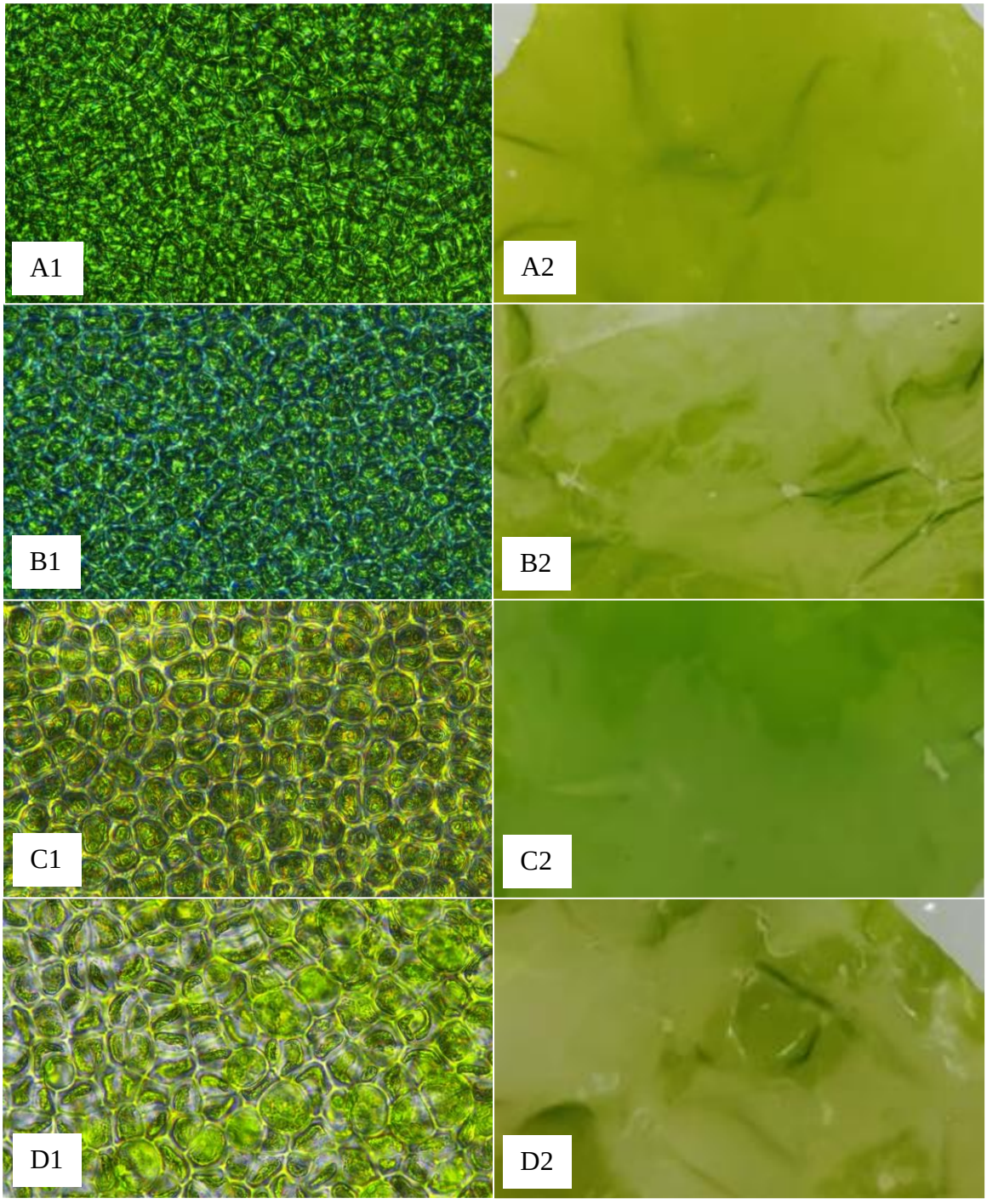

Figure 3.17: Ulva sp. tissue samples with vegetative state (A) and the observed reproduction state in harvest 1 (B), 2 (C) and 5 (D). Images taken at 40x magnification (1; left column) and the visual view by eye from the same tissue (2; right column).

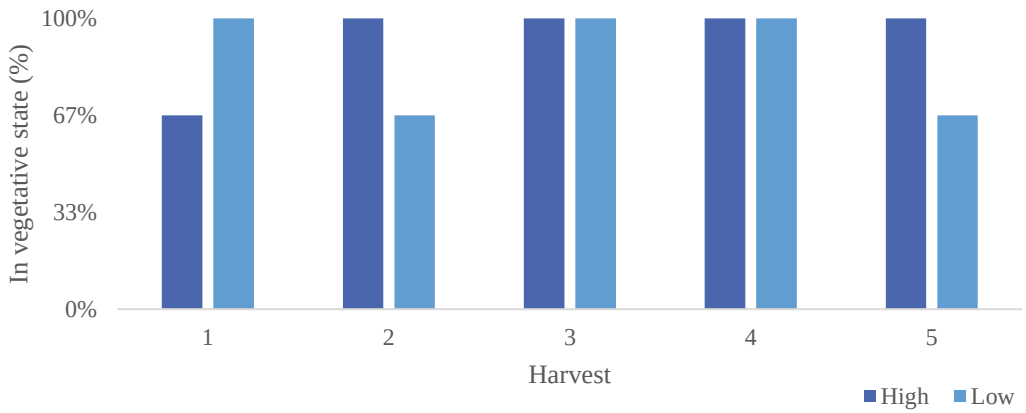

Figure 3.18: Percentage of samples in vegetative state (compared to reproduction state) for each harvest in IRAS High-F and Low-F (n = 3).

3.2.8 Additional tissue observations

Important to mention is that in IRAS High-F *Ulva* sp. developed a more fragile and softer tissue compared to the more plastic-like structure in Low-F. In harvest 3 the tissue in IRAS High-F started to develop curling thalli and was sometimes covered with epiphytes (Figure 3.19).

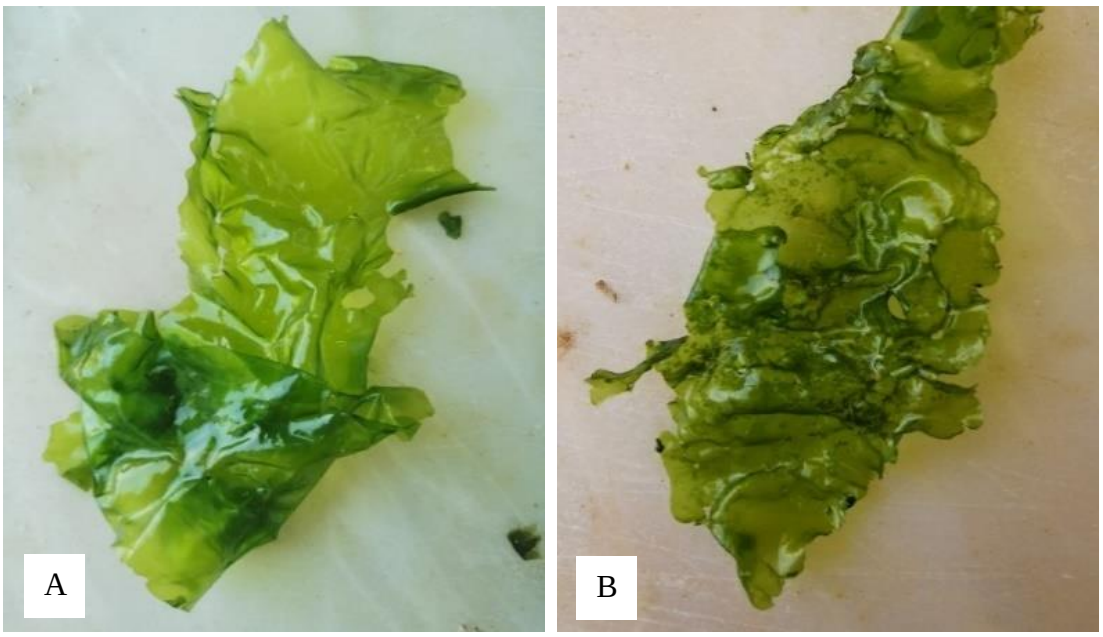

Figure 3.19: Comparison of healthy Ulva tissue from IRAS Low-F (A) and curling tissue sample covered with epiphytes from High-F (B). Observed in harvest 3.

4. Discussion

As water quality is extremely important in aquaculture, nitrogen treatment capacity is the limiting factor of total fish production in IRAS (Besson et al., 2014). Bioremediating nutrients by seaweeds in aquaculture farming could alleviate the nutrient load in the water, thereby promoting productivity in both fish and seaweed. To optimize IRAS, the primary objective of this study was to maximize N removal by *Ulva* sp., while simultaneously increasing biomass yield. The current study indicates that the *Ulva* sp. biofilter was effective in transforming nutrients from the water into biomass and altering water quality parameters. This means that seaweed is a valuable alternative to filter water in a sustainable manner and increases the profitability of the more expensive recirculating systems. Nevertheless, the data from the experiment also suggests that seaweed performance is affected by factors like bacterial pressure in the IRAS, so it is important to do more research of controlling nutrients in the system by additional biofilters with different nutrient preferences from the *Ulva*, especially when there is a high nutrient input.

4.1 Water quality

4.1.1 General environmental conditions

During the experiment the fish densities were reduced to ensure proper water quality for the fish. For this reason, the data was analyzed separately. Cycle 1 had a fish density of 20 and 10 kg fish m^{-3} for IRAS High-F and Low-F, respectively, with 2 harvests. For Cycle 2 the densities were reduced to 10 and 5 kg fish m^{-3} with 3 harvests to meet the capacity of the IRAS to treat the water in the system with the high fish density. Average ammonia level in IRAS High-F was 1.26 mg L^{-1} in Cycle 1 and 0.43 mg L^{-1} in Cycle 2. For IRAS Low-F this was 0.23 mg L^{-1} in Cycle 1 and 0.07 mg L^{-1} in Cycle 2. Flow rate was finally adjusted to 450L h^{-1} in the seaweed tanks as higher rates caused overflowing, but this limited the N supply to the seaweeds.

In Cycle 1, the average daily water temperature and photon irradiance during daylight in the seaweed tanks were 19.7°C and 287.8 W m^{-2}, respectively. For Cycle 2 these average parameters were 23.7°C and 320.0 W m^{-2}, respectively. Water temperature and light intensity have been known to influence growth. The optimal water temperature for *Ulva* sp. is 25°C (Mantri et al., 2010). Together with a higher photon irradiation for photosynthesis we can assume that Cycle 2 offered improved conditions for seaweed growth compared with Cycle 1.

4.1.2 Microbial community

IRAS High-F had significantly more heterotrophic bacteria abundance than IRAS Low-F, in specific 86% and 74% for Cycle 1 and 2, respectively. After the fish densities were reduced, we counted an average of 2.37 x 10^{10} and 1.37 x 10^{10} cells L^{-1} of heterotrophic bacteria counted in IRAS High-F and Low-F, respectively. This is a fairly high number, as in natural waters their count ranges from 10^{8} to 10^{10} cells L^{-1} (Wright, 1978). Nevertheless, their abundance could be beneficial in IRAS. A study by Tupas & Koike (1991) demonstrated that heterotrophic bacteria actively assimilate ammonium and simultaneously mineralize dissolved organic nitrogen, also in dark conditions, implying the importance of their populations for ammonium removal and regeneration. They counted an average of 10^{9} cells L^{-1} in their nitrogen-enriched samples, ten times less than in our samples. Even though the bacteria support ammonia removal in the system, their metabolic processes consume oxygen. This consequently inhibits nutrient bioremediation, because oxygen is required for mechanisms like ammonia oxidation (Ohashi et al., 1995). When heterotrophic bacteria abundance increased, we also counted more bacterivores. Many bacterivores in our water samples were mixotrophic flagellates and cryptophytes. This increased abundance was expected, as heterotrophic flagellates are the most important grazers of bacteria (Boenigk & Arndt, 2002). A high cryptophyte abundance indicates poor water quality, as their dominance increases with a higher level of NH_4^+ (Kang et al., 2021). We also observed ameboid protists in the water samples, which indicates environmental stress like highly eutrophicated water and/or low oxygen levels (Regalado et al., 2018). We speculate that oxygen enrichment would be effective to support ammonium reduction processes in the water.

We found no significant difference in pico- and nanophytoplankton between the two systems, although the phytoplankton groups (picoeukaryotes, diatoms and cyanobacteria) increased with rising temperatures. Temperature is one of the most important factors that influence composition and metabolism in planktonic communities. Higher temperatures stimulate primary production, and the bacteria feed on the dissolved organic matter that is released by seaweeds and phytoplankton (Liang et al., 2021; Pan et al., 2007). Furthermore, there was a development of filamentous bacteria, and conditions like long solids retention time, DO deficiency, and high temperature enhance their growth (Liu & Liu, 2006). We observed an accumulation of organic matter on the Bioballs, mainly in IRAS High-F, and a high concentration of suspended solids supports a stronger growth

of opportunistic heterotrophic bacteria that compete with nitrifying bacteria for oxygen and space (Ray et al., 2010). Abundant organic matter and increasing water temperature can lead to fast mineralization that increase inorganic nitrogen compounds in the water. Higher ammonia levels motivate the proliferation of opportunistic pathogens that can cause disease development (Infante-Villamil et al., 2021). Hence, DO greatly influences the microbial community diversity and relative abundance, and the oxygen demand depends on the microbial community that can change rapidly depending on the available resources (Bucking, 2017; Li et al., 2020; Pomeroy & Wiebe, 2001). This emphasizes the importance to keep DO high. Burford et al. (2003) suggests that healthy microbial dynamics in a closed system can have a balanced oxygen production and consumption while supporting bioremediation of the water, because bacteria and phytoplankton can take up considerate amounts of ammonium and nitrate as well. To further improve the water quality we suggest to investigate the implementation of Biofloc Technology, a technique that is already successfully implemented in many shrimp farms (Khanjani & Sharifinia, 2020). It promotes microbial growth by adding external carbon sources to reduce the nutrient load in the water.

4.2 *Ulva* performance

4.2.1 Growth rate and yield at different fish densities

In our experiment, *Ulva* performed better in IRAS Low-F. The average SGR and yield in the last cycle were 26.7% day^{-1} and 1064.8 g FW m^{-2} day^{-1}, respectively, while for IRAS High-F these values were 21.4% and 770.5 g. This is a surprising result, as we hypothesized that IRAS High-F would perform better because of the higher nutrient load in the water. A possible explanation can be that there was a pressure of disease in IRAS High-F because we observed that *Ulva* tissue started to curl and started to develop parts that were covered with epiphytes halfway through the experiment. The presence of epiphytes is an indication that *Ulva* was stressed and had a suppressed growth rate (Neori et al., 2004). The observed reproduction states did not seem to significantly affect performance. Another theory is that the seaweeds in IRAS High-F needed to adapt to the much higher nutrient load than in a natural setting. Ale et al. (2011) showed that *Ulva* has a favorable growth response to ammonium compared to nitrate as nitrogen-source, as nitrate requires metabolic energy to take up. Steffensen (1976) found that above the optimum levels of 0.6 g m^{-3} NO_3-N results in limiting growth of *Ulva lactuca*. Lundberg et al. (1989) even found indications that exposure to high nitrate concentrations inhibit

phosphorus intake by *Ulva* spp., thereby limiting growth. Furthermore, Chatzoglou et al. (2020) observed that although *Ulva* bioremediates ammonia and nitrate, they can also enrich the water with phosphates. Nederlof et al. (2022) found that a high orthophosphate concentration (0.9 mmol L^{-1}) is toxic to *Ulva* spp. Studies about the inhibition effects of ammonia on *Ulva* are not well documented, as reduced growth is often attributed to limiting resources like light. To optimize the IRAS, we suggest studying at which levels ammonia limits *Ulva* growth and considering adding an additional biofiltration unit of seaweed species that prefer nitrate and phosphates. The nutrients water samples yet to be analyzed should provide some clarification on this.

The performance of *Ulva* was slightly better in Cycle 2 even though there was a reduced N supply, possibly related to improved environmental conditions like increased light exposure, higher water temperature, and reduced bacterial pressure. Also, the low fish density in Cycle 1 is the same as the high fish density in Cycle 2. During the full experiment, a drop in performance was observed for harvest 3, which was expected as the fish densities were reduced in the IRAS after harvest 2. The highest average SGR and yield were observed for harvest 5, the last harvest. As water temperature and photon irradiation were less than in the previous two harvests, we speculate that the *Ulva* was still adapting to the cultivation environment. We speculate that the performance of *Ulva* in IRAS is strongly related to photon irradiation, water temperature, adaptation, and health status of the *Ulva*, and that the nutrient load was in both systems exceeding the uptake capacity of the seaweed unit. The length of the experiment should be increased to determine the relevant factors that caused the difference in performance.

4.2.2 Growth rate and yield at different stocking densities

We also found that growth of *Ulva* decreases in this system when tested stocking density increased. In Cycle 2, the stocking density treatments 1, 2, and 3 kg m^{-2} had an average SGR of 31.5, 23.1, and 17.4% d^{-1}. Both IRAS in Cycle 1, and IRAS Low-F in Cycle 2, yield did not significantly differ between the stocking density treatments. However, in IRAS High-F in Cycle 2, yield was only significantly higher for *Ulva* stocking density 2 in higher fish density (891.0 g FW m^{-2} / 139.7 g DW m^{-2}) than *Ulva* stocking density 1 (603.7 g FW m^{-2} / 94.6 g DW m^{-2}). A study by Shpigel et al. (2019) of N uptake by algae in an IMTA system also experimented with different *Ulva* stocking densities. They found that 1 kg FW m^{-2} for *Ulva lactuca* shows a higher average yield and specific growth rate (SGR) compared to increased densities with an average of 18% d^{-1} and 25.1 g DW m^{-2} d^{-}

[1], while a stocking density of 3 kg FW m^{-2} show more nitrogen assimilation per g FW compared to lower densities. The performance in our study was much higher than in their study, most likely caused by a higher nutrient load because of water circulation. There is still a lack of research in the growth performance of *Ulva* in different stocking densities in outdoor RAS, as almost all research is focused on bioremediation potential and the influence of environmental factors on production. N removal could be optimized when a stocking density with maximal biomass yield is used in the system.

4.2.3 N removal by *Ulva* biomass

N yield is used to estimate biofiltration, as it reflects on the N removal by the seaweeds in the system. Cohen & Neori (1991) found that N-content significantly increased with a higher stocking density, but we found no significant effect of *Ulva* stocking density on N content. We found however that *Ulva* in IRAS significantly increases in N content, and that a higher fish density results in higher N content. A higher N content means they remove more N per unit of biomass. The highest N content of 3.82% was seen when average ammonia level was 1.26 mg L^{-1} and decreased to 2.94% when fish density was reduced from 20 to 10 kg m^{-3} with an average ammonia level of 0.43 mg L^{-1}. N yield however was higher at the lower fish density in Cycle 2, indicating that a higher biomass yield leads to higher N removal. In the experimental IRAS we measured biomass yields between 119 – 167 g DW m^{-2} d^{-1}, more than 2 times as high as reported in other experiments with similar set-up (reviewed in Neveux et al., 2018). DW was calculated differently as they used the fresh weight to dry weight ratios instead of a linear regression equation that we used in this study. It is important to note there might have been significant errors introduced in all the FW measurements by the excess water removal method used in this study. In an integrated flow-through fish/seaweed aquaculture, maximum yield values were estimated to be 52 g DW m^{-2} d^{-1} and N content was averaged to be 5.25% DW for *Ulva rigida* (Mata et al., 2010). They state that with an increased light exposure/culture volume ratio, the ultimate *Ulva* stocking density becomes higher, consequently enhancing nutrient biofiltration and biomass yield. We expect that the transparency and volume to surface of the seaweed tanks were inhibiting growth potential, which explains why there was no significant difference found between the *Ulva* stocking densities.

4.2.4 Water quality and *Ulva*

We only looked at differences in fluctuations in DO, as the addition of pure oxygen was not easy to control and might have been different in the two IRAS. During the day there was an increase in pH and decrease in oxygen in the seaweed tanks. The lower DO was the opposite from what was expected since *Ulva* is known to be strong in both oxygen and pH generation. Photosynthesis is its only mechanism to assimilate energy and this can normally be seen in low oxygen saturation and pH early morning and maximum levels in the afternoon (Axelsson, 1988; Shahar & Guttman, 2021). As photosynthesis produces oxygen, we hypothesized that IRAS High-F with a higher nutrient load than IRAS Low-F would give the highest *Ulva* growth rates and subsequently would lead to a higher DO in the system, but this was not found. Our results were supported by Gao et al. (2016), where they stated that higher growth rates do not necessarily increase photosynthetic rate. In our experiment, unlike *Ulva* performance, DO and pH were quite similar between the harvests, with the exception of the first harvest. Only pH in IRAS High-F was significantly lower than IRAS Low-F in both cycles. The lower pH indicated that there was more CO_2 production in IRAS High-F, and this was not counterbalanced by the oxygen production by *Ulva*. Our data suggests that oxygen and pH are more related to seaweed biomass instead of strength of production, and this limits the capacity of the seaweed unit to treat recycled water in IRAS. Similar to seaweed cultivation, the microbial community diversity has a strong impact on the nutrient removal mechanisms, including nitrification, ammonification, and denitrification that contribute to higher water quality (Nie et al., 2020; Wang et al., 2011). In a study by Chatzoglou et al. (2020) *Ulva* was co-cultured in a RAS setting similar to this experiment and they concluded that *Ulva* leads to enhanced pH and O_2, and reduced CO_2, TAN and nitrates. We found similar shifts in these water quality parameters. Moreover, they found positive effects on the growth and quality of the co-cultured fish in their experiment. We speculate that to further increase the water quality in IRAS, it is important to study how to support a healthy community of phytoplankton and bacteria together with seaweed cultivation.

4.3 Final conclusions

This study showed that using *Ulva* sp. as a biofilter unit in IRAS with *Sparus aurata* has a positive effect on seaweed performance and water quality. Our study suggests that using the stocking density 2 kg FW m^{-2} will be most beneficial in IRAS compared to 1 and 3 kg. Since we have found a higher performance in the IRAS with the lower nutrient load

against expectations, we recommend investigating which factors can inhibit and promote the growth of *Ulva* for further optimization of IRAS. Before adoption by commercial aquaculture farms, more studies should follow in optimizing seaweed culture methods in IRAS to improve nutrient removal. The next step should involve researching combinations of biofilters with different nutrient preferences to increase its capacity to improve water quality parameters.

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