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## **Integrating seaweed in a marine fish RAS**



Universidade do Algarve  
Faculdade de Ciências e Tecnologia

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**Integrating seaweed in a marine fish RAS**

Master's degree in Aquaculture and Fisheries:

Specialization in Aquaculture

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Faculdade de Ciências e Tecnologia  
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## **Abstract**

Aquaculture is, nowadays, one of the sectors with a crucial impact on the human food supply. Sustainability in this sector has become a primary goal and is directly connected to recirculating aquaculture systems. These systems hold great promise for the future, but improvements in water quality are needed due to the high accumulation of metabolic waste. Water quality may be improved by integrating macroalgae into these systems, as they have excellent bioremediation capabilities. This study aimed to implement an Integrated Recirculating Aquaculture System (IRAS) with *Ulva* sp. and fish to assess macroalgae bioremediation capacity and improve water quality and fish performance in comparison to a fish Recirculating Aquaculture System (RAS). Macroalgae capacity in bioremediation highly depends on their growth and nutrient availability in the system. So, this work included sampling of dissolved nutrients and weekly samplings for assessing biomass production, at different densities (1.0, 1.5, and 2.0 kg FW m<sup>-2</sup>), and assessment of nitrogen content in the macroalgae produced. Results showed that the highest growth rate was found for *Ulva* sp. stocking density 1.0 kg FW m<sup>-2</sup>, while N yield did not differ between the stocking densities tested. The *Ulva* sp. improvement in water quality parameters was not significant when compared to RAS treatment. Fish performance was not significantly different between treatments and no differences were observed in hematological and histological parameters. The gills presented some anomalies but were classified with minor signs of pathology. The results demonstrate that lower *Ulva* sp. stocking densities resulted in higher growth, indicating that this macroalgae can be a potential candidate for IRAS, but more research is needed to use the bioremediation capability to improve water quality in these systems.

## **Keywords**

IRAS, bioremediation, macroalgae, fish performance, water quality

## Resumo

A aquicultura tem-se revelado nos últimos anos como o setor alimentar com maior crescimento no mundo. A crescente procura tem levado a práticas mais sustentáveis na aquicultura. O desenvolvimento destas práticas tem aumentado ano após ano, com otimizações dos sistemas existentes e a integração de novas espécies em sistemas já utilizados a serem soluções para uma aquicultura mais sustentável.

Os Sistemas de Aquicultura de Recirculação (RAS) oferecem soluções ao nível da conservação dos recursos hídricos, redução da poluição e aumento da eficiência na produção de organismos aquáticos. A utilização destes tipos de sistemas tem aumentado gradualmente ao longo dos anos. A sua capacidade em aumentar a variabilidade e sustentabilidade de espécies em apenas um cultivo torna-se altamente benéfica ao garantir as mesmas ou melhores condições para o cultivo base, permitindo atingir diferentes mercados pela integração de novas espécies de diferentes níveis tróficos nestes sistemas recirculados (IRAS). A integração dessas novas espécies pode também ter um impacto positivo na qualidade da água, ou até nas espécies integradas no sistema, permitindo assim um desenvolvimento mais sustentável do sistema em si.

As principais dificuldades associadas ao RAS advêm do custo, da experiência necessária para operar esses sistemas e, principalmente, da gestão da qualidade da água. A troca reduzida de água causa acumulação de nutrientes dissolvidos dentro do sistema e, se não forem devidamente removidos, podem tornar-se um problema para o sistema. Em casos particulares, a produção de elevados níveis de amónia, nitritos e nitratos pode causar sérios problemas nos peixes, originando doenças, danos físicos ou até a morte, em casos extremos de presença desses compostos. A manifestação desses problemas pode ser observada pela análise dos parâmetros hematológicos do peixe ou pela observação das brânquias em microscopia. A observação do sangue do peixe consegue fornecer uma imagem dos processos endócrinos, imunológicos, reprodutivos, e genéticos em relação às condições ambientais a que foram submetidos. O hematócrito e a hemoglobina são indicadores importantes do transporte de oxigénio no peixe, permitindo fazer comparações entre o oxigénio transportado e a performance do peixe num sistema durante um determinado espaço de tempo.

A integração de macroalgas em RAS pode servir como solução para a remoção de compostos como amónia, nitratos e fosfatos da água. As macroalgas têm um elevado potencial biorremediador, a sua facilidade de absorção de nutrientes como o azoto e o fósforo da água potencia a redução da acumulação desses nutrientes no sistema. As macroalgas durante o seu processo de fotossíntese não só libertam oxigénio (O<sub>2</sub>) na água, podendo aumentar os níveis de oxigénio dissolvido e assim melhorar as condições de vida dos organismos neste sistema, como também reduzem os níveis de dióxido de carbono (CO<sub>2</sub>), que são altamente prejudiciais para os peixes. A integração das macroalgas potencia uma prática promissora para melhorar a qualidade da água, promover a sustentabilidade e aumentar a eficiência do sistema RAS.

Este estudo tem como objetivo comparar um sistema base RAS com um sistema integrado com o género *Ulva* sp. (IRAS). Como objetivo específico, pretende comparar entre sistemas o nível de qualidade da água, performance dos peixes e a própria capacidade de biorremediação da macroalga, assim como o seu crescimento a diferentes densidades de cultivo. Com a integração da macroalga do género *Ulva* sp. num sistema em recirculação com dourada (*Sparus aurata*) (IRAS), procuram-se resultados positivos no crescimento e desenvolvimento dos peixes e das macroalgas.

Para alcançar este objetivo, foram usados dois sistemas distintos: RAS e IRAS, onde ambos foram submetidos às mesmas condições, incluindo igual densidade de peixes ( $12 \text{ kg m}^{-3}$ ), diferenciando-se apenas o IRAS, onde foi introduzida a espécie *Ulva* sp. Temperatura, oxigênio, pH e salinidade foram medidos e injeções de oxigênio foram efetuadas em ambos os sistemas, de forma a garantir condições adequadas para o desenvolvimento de ambas as espécies. A *Ulva* sp. foi introduzida a três densidades diferentes ( $1.0$ ,  $1.5$ , e  $2.0 \text{ kg peso fresco m}^{-2}$ ) e amostragens semanais foram efetuadas para verificar o crescimento e a biorremediação de nitrogênio para as três densidades cultivadas. A macroalga demonstrou uma taxa de crescimento (SGR) significativamente maior na densidade mais baixa. Os valores de absorção de nitrogênio não variaram entre densidades, mas tiveram um aumento significativo quando medidos no fim do ensaio em comparação com os medidos inicialmente.

Para avaliar e comparar a qualidade da água entre os dois tratamentos ao longo das semanas e ao longo de um dia do ensaio, foram efetuadas recolhas de água semanalmente nos dois tratamentos, e na terceira, quarta e quinta semana foram feitas recolhas a cada duas horas, entre as 06h00 e as 22h00. A ausência de diferenças significativas nos nutrientes entre os dois sistemas revela que a qualidade da água foi similar entre ambos os tratamentos na análise por semanas. No entanto, na análise de duas em duas horas, os valores de amônia e nitritos diferiram entre os tratamentos, possivelmente devido a períodos de excreção dos peixes a coincidir com as últimas horas de recolha de amostras. Na análise semanal, foram verificadas variações significativas na primeira semana e nas últimas semanas de ensaio para  $\text{NH}_3$  e  $\text{NO}_3^-$ , tais oscilações podem estar associadas ao aumento de temperatura verificado na fase final do ensaio.

Era esperado que a presença da macroalga tivesse um impacto bioremediador significativo nos nutrientes dissolvidos e possivelmente na condição do peixe. Para avaliar esses resultados, foram realizadas análises hematológicas e observações histológicas nos peixes no final da experiência. No entanto, não foram identificadas diferenças significativas na concentração de nutrientes dissolvidos, e os resultados indicaram um impacto não significativo nas análises histológicas e hematológicas dos peixes. Apesar da ausência de diferenças entre os dois sistemas, observou-se um crescimento em peso e comprimento total nos peixes em ambos os tratamentos.

Os resultados sugerem que a *Ulva* sp. é uma macroalga que se integra eficientemente com os sistemas recirculados. Embora não tenham sido observadas mudanças significativas na qualidade da água, ambos os tratamentos demonstraram um crescimento e uma boa condição do peixe. Com mais estudos neste tema, poderá ser alcançada uma proporção adequada entre a biomassa inicial de macroalga e de peixes no sistema, o que poderia resultar em impactos significativos na performance dos peixes e na produção de *Ulva* sp.

#### **Palavras-chave:**

IRAS, biorremediação, macroalga, condição do peixe, qualidade da água

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## **List of abbreviations**

d Day

DO Dissolved Oxygen

DW Dry Weight

EPPO Estação Piloto de Piscicultura de Olhão

FW Fresh Weight

Hgb Hemoglobin

HTC hematocrit

IPMA Instituto Português do Mar e da Atmosfera

IMTA Integrated Multitrophic Aquaculture

IRAS Integrated Recirculating Aquaculture System

K Condition Factor

N Nitrogen

Nc Nitrogen content

RAS Recirculating Aquaculture Systems

SD Standard Deviation

SGR Specific Growth Rate

TAN Total Ammonia Nitrogen

TGC Thermal-unit Growth Coefficient

UAlg University of Algarve

WG Weight gain

## **1. Introduction**

### **1.1 Aquaculture**

Nowadays aquaculture is considered to have a crucial role in human food supply. Between 1961 and 2019, the apparent global consumption of aquatic foods grew at an average annual rate of 3.0%, nearly twice as fast as the global population's annual growth rate of 1.6% during that time (FAO, 2022). Compared to 2019, the production of animal species in aquaculture increased globally by 2.7% in 2020. Additionally, the production of algae, primarily marine macroalgae or seaweeds, increased by 500,000 tonnes in 2020, a rise of 1.4% from the 34.6 million tonnes produced in 2019 (FAO, 2022). Portuguese fish consumption per capita in 2018 was 61.5 kg, making Portugal one of the highest consumers worldwide.(FEAP, 2021). A sustainable aquaculture can respond to the high demand for fish consumption while respecting ecological parameters, but a higher development or progress is necessary, increasing knowledge and production (FAO, 2022). However, Portugal exhibits slower growth and advancement when compared to European aquaculture output, contributing to less than 1% of the entire aquaculture production in terms of volume (EUMOFA, 2021).

The adoption of advanced aquaculture farming systems and other technological innovations is demonstrating a positive influence on aquaculture outcomes, including production, investment, and market potential while emphasizing the need for collaborative efforts between farmers, scientists, and the international donor community to promote eco-friendly practices and the development of healthy, fast-growing animals (Jena et al., 2017). One of the most used systems showing potential in terms of quality and efficiency are Recirculating Aquaculture Systems (RAS).

### **1.2 Recirculating Aquaculture Systems**

One of the main concerns in aquaculture production is the water consumption associated with wastewater release. Effluents play a crucial role in the impacts to the environment, depending on the type of system used. Recirculating Aquaculture Systems (RAS), as defined by Martins et al. (2011) are land-based aquatic production systems where the water is (partially) re-used after mechanical and biological treatments to reduce the consumption of water and energy and the release of nutrients into the environment.

RAS were developed as a technology for intensive fish farming, used mainly when water availability is restricted: they enable up to 90–99% of the water to be

recycled, through the utilization of many different components, as for example mechanical filters, biofilters, oxygenation, solids removal, and disinfection systems, which work together to create a closed-loop system (Timmons & Ebeling, 2010). These systems offer advantages in terms of reduced water consumption, improved opportunities for waste management, nutrient recycling, and biological pollution control (Verdegem et al., 2006), allowing the operator greater control over the environmental and water quality parameters, thus enabling optimal conditions for fish culture (Heinen et al., 1996). In RAS the movement of water will contribute to the even distribution of environmental factors, such as temperature, oxygen, pH, and others, and also to spread the feed, impurities and fish metabolic waste (Diansari et al., 2013; Tanjung et al., 2019). In terms of hygienic conditions and disease management, there are some authors (Summerfelt et al., 2009) defending that RAS assure better control of these factors, while others point out that it is necessary a very careful management and there are difficulties in treating the diseases, besides high capital and operational costs (Schneider, 2006).

### **1.3 Water quality in RAS**

Water quality in RAS is dependent on a wide range of factors, and each factor needs to work properly to guarantee good water quality. Nitrogen (N) is a parameter that needs to be taken in account in any aquaculture system. Ammonium ( $\text{NH}_4^+$ ), which is present in large concentrations and is harmful to fish, is a by-product of animal catabolism or the waste from the consumption of feed (Eshchar et al., 2006).  $\text{NH}_3$  (ammonia) and  $\text{NH}_4^+$  (ammonium) are in balance in water depending on the temperature and pH (Timmons & Ebeling, 2007). Total ammonium nitrogen (TAN) includes the two forms. Although both  $\text{NH}_3$  and  $\text{NH}_4^+$  may be hazardous to fish, unionized ammonia is the more dangerous form because it is uncharged and lipid soluble and can cross cellular membranes more easily than the charged and hydrated  $\text{NH}_4^+$  ions (Colt, 2006). Nitrite can also be toxic to the fish and significant issues arise in the intense culture of commercial fish species due to elevated nitrite concentrations (Svobodová et al., 2005). Also, nitrate levels in high amounts can be critical due to its reaction with hemoglobin, which results in a lack of oxygen to the fish and ultimately their death (Camargo et al., 2005). In general, values of  $\text{NH}_3$  over  $0.025 \text{ mg L}^{-1}$  and  $\text{NO}_2$  (Nitrite) over  $0.5 \text{ mg L}^{-1}$  can be toxic to the fish, while values of  $\text{NO}_3$  (Nitrate) over  $300 \text{ mg L}^{-1}$  can be harmful to the fish (Bregnballe, 2022).  $\text{NH}_3$ ,  $\text{NO}_2$ , and  $\text{NO}_3$  levels in an aquaculture system are influenced by various factors, including fish species and size, fine solids, refractory organics, surface-active

compounds, metals, water quality parameters (such as pH, temperature, and oxygen concentration), as well as the type of biofilter present in the system and the frequency of water exchange (Colt, 2006; Kroupova et al., 2005; Pavlidis & Mylonas, 2011).

Filters in RAS are essential for improving the water's quality. Mechanical filters work to remove relatively big coarse of contaminants such as faeces, mucus and leftover feed while bacterial biofilter are needed to oxidize toxic ammonia and nitrite (Guenard, 2021). Chemical filters, like charcoal, work to remove particles that cannot be handled by mechanical filters (Tanjung et al., 2019). The colonization of the bacterial biofilter is an important process because colonized bacteria will allow the reduction of ammonia from the system by the process of nitrification. This process turns hazardous ammonia nitrogen ( $\text{NH}_3$ ) into  $\text{NO}_2^-$  and then to  $\text{NO}_3^-$ . A suitable surface (biofilter medium) on which the bacteria can grow, the maintenance of normal water temperatures, and high water quality are necessary for the process (Helfrich & Libey, 1990).

Nowadays, the improvement of water quality in aquaculture systems is prioritized through innovative approaches such as Integrated Multi-Trophic Aquaculture (IMTA) and Integrated Recirculating Aquaculture Systems (IRAS). By strategically integrating biofilters such as plants or algae, these systems effectively minimize nutrient discharges, such as nitrates and phosphate, and enhance overall water quality, promoting sustainable and efficient aquaculture practices (Turcios & Papenbrock, 2014).

#### **1.4 Fish performance in RAS**

The overall well-being and performance of fish under intensive culture conditions depend on factors such as water quality and health conditions (Oliva-Teles, 2012). In RAS water quality is a factor of major importance, concerning that the presence of solids in the water and bad nitrification processes can significantly heightens stress levels and detrimentally affects the performance of fish (Badiola et al., 2012). The performance of the fish, encompassing various physiological and behavioral aspects, is intricately connected with histopathological alterations reported in several organs. These alterations not only provide insights into the health of individual fish but also serve as valuable biomarkers for distinguishing pollution levels among various aquatic environments (Nascimento et al., 2012). Gills are essential organs for fish respiration and ion regulation. However, significant changes in environmental conditions can significantly impact these organs (Nogueira et al., 2008). The concept of gill health is deduced by contrasting typical

and atypical anatomical structures, such as epithelial hyperplasia (increased cell production), lamellar fusion, cellular anomalies (degeneration and necrosis), hypertrophy, oedema, eosinophilic granular cells, inflammation, circulatory damage, and congestion of secondary lamellae, and the presence, extent or severity of these pathologies are important tools for assessing the performance of fish under different conditions (Araújo-Luna et al., 2018; Foyle et al., 2020). Besides the gills, the blood can provide significant information regarding the physiological status of the fish. Examining fish blood can provide a thorough picture of endocrine, immune, reproductive, and genetic processes in relation to environmental situations (Seibel et al., 2021). Regular hematological analyses include measures of biochemical indices, such as plasma component concentrations or activities, as well as red blood cell parameters (Witeska et al., 2022). The hematocrit percentage, hemoglobin, and erythrocyte count are important parameters to analyze fish performance because they are reliable indicators of the fish's ability to transport oxygen, allowing relationships to be established between the oxygen concentration in the experimental setting and the performance of those fish's (Lamas et al., 1994). Water quality not only shapes the outcomes of haematological and histopathological analyses but also directly influences the physiological performance of fish in RAS. An optimal water quality, characterized by a well-functioning nitrification process, well-established feeding practices, sufficient oxygen levels, appropriate temperature, and careful design and management of stocking densities, is essential for maintaining excellent fish performance (Helfrich & Libey, 1990).

### **1.5 IMTA and IRAS**

Integrated Multi-Trophic Aquaculture (IMTA) is a sustainable farming method that involves cultivating multiple species from different trophic levels, each with specific ecological functions, within a shared aquatic environment. By strategically combining species with complementary nutrient requirements, IMTA aims to optimize resource utilization, minimize waste, and enhance overall ecosystem health and productivity (Chopin, 2010; Science, 2020). This IMTA concept can be extremely flexible, can be applied to open-water or land-based, marine or freshwater systems (FAO, 2009). Like any other system, there are advantages and disadvantages. IMTA systems can provide effluent bio-mitigation, increase profits through diversification and improve disease management by including certain types of species. On the other hand, IMTA is associated

with lower productivity than monocultures, can cause some public perception issues and a very good management is necessary (Yadav & Sharma, 2018).

Integrated recirculating aquaculture system (IRAS), is the result of combining RAS and IMTA and can be the solution to combine nutrient recycling, reduce water use and nutrient discharge while obtaining products for different markets (Neori et al., 2000, 2017). Since IRAS is a type of IMTA, it shares similar advantages and disadvantages. IRAS systems tend to accumulate solids and nitrates, but through the integration of extractive species, these by-products can be reduced. An example of species that can be integrated into IRAS are macroalgae, which may have an important role in this type of systems, since as primary producers have the ability to add oxygen to the water, while removing dissolved nutrient wastes from the system (Neori, 2008).

## **1.6 Macroalgae**

Macroalgae can be found in a wide variety of habitats and conditions as marine or freshwater, on surfaces of soil and rocks (H. M. D. Wang et al., 2015). The term seaweed in this work refers solely to marine macroalgae. Seaweeds are a diverse group of algae, consisting of different divisions, namely Division Rhodophyta (red), Division Chlorophyta (green), and Division Ochrophyta, specifically Class Phaeophyta (brown) (Paul et al., 2006)

Macroalgae have a wide range of applications nowadays, including human food, agriculture, cosmetics, and pharmaceuticals (Leandro et al., 2020). Asian countries are particularly known for their consumption of seaweed due to its nutritional richness and low-calorie content (Leandro et al., 2020). In agriculture, macroalgae find multiple uses such as animal feed, biostimulation for plant growth, and their antibacterial, antifungal, antiviral, and bioinsecticide properties (Hamed et al., 2018; Lawton et al., 2017). Seaweeds are also highly valued in the cosmetics industry due to their abundance of vitamins, minerals, amino acids, antioxidants, and essential fatty acids, which contribute to their effectiveness in cosmetic products (Pereira, 2018). Moreover, the pharmaceutical industry recognizes macroalgae as a valuable field of application, given their ability to adapt to various environmental stresses and threats, leading to extensive research on their pharmacological effects (Leandro et al., 2020).

## 1.7 Macroalgae in IRAS

Marine macroalgae have been recently used in IRAS, since they may improve water quality (Ramli, 2018) and help to control bacteria in the system (Natrah et al., 2011; Tendencia et al., 2015).

Bioremediation is a recognized process that utilizes various biological agents such as bacteria, fungi, higher plants, algae, and cyanobacteria to eliminate environmental contaminants and restore land or water quality (Carter et al., 2006). Seaweed is an appealing and suitable resource for promoting sustainable aquaculture practices, since the capacity of macroalgae to adapt to the presence of anthropogenic nutrients, particularly nitrogen and phosphorus inputs, enhances their effectiveness as a bioremediation solution (Neori et al., 2004). The performance of a marine macroalgae biofiltration depends on several parameters, some of which are controllable (*e.g.*, culture structure design, stocking density, algal strain, nutrient load and hydraulic regime), while others (*e.g.*, climate, weather, pests and light conditions) are not (Ben-Ari et al., 2014).

RAS require good water quality, and macroalgae have mechanisms that assure this quality. Macroalgae role in the removal of ammonia and nitrate is crucial, for example, the *Ulva* sp. can be extremely efficient in nutrient uptake, but its efficiency depends on the type of system and how optimized it is (Anfbal et al., 2014).

## 1.8 Target species in the trial

### 1.8.1 *Ulva* sp.

Seaweed from the genus *Ulva* is a green alga, whose main habitat is the upper eulittoral zones of subtropics, especially on river mouths where diurnal fluctuation in salinity is high (Bast, 2014). Due to their rapid growth and rapid uptake of organic matter, these are ideal candidates for use in bioremediation on marine macroalgae farms (Henriques et al., 2017). According to Al-Hafedh et al. (2015), positive results in TAN and phosphate removal were found when *Ulva* was integrated with tilapia (*O. spilurus*) in an aquaculture system, with removal rates of TAN ranging from 0.26 to 0.31 g m<sup>-2</sup> day<sup>-1</sup> and phosphorus from 0.32 to 0.41 g m<sup>-2</sup> day<sup>-1</sup>. The uptake of nutrients is associated with rapid growth and an increase in the biomass resulting from this absorption has been verified in different studies under different conditions (Al-Hafedh et al., 2015; Cahill et al., 2010).

*Ulva*, a genus known for its tolerance to temperature and salinity fluctuations, relies on a reliable source of seedlings and consistent biomass production for its sustainable long-term cultivation (Carl et al., 2016).

*Ulva* sp. is a sustainable and valuable biomass with abundant bioactive compounds, making it suitable for various industries. Its exceptional growth rates, efficient nutrient assimilation, and adaptability to changing environmental conditions make *Ulva* sp. an ideal candidate for integration into RAS, particularly as a biological filter to effectively mitigate eutrophication and nutrient removal from water (Mabeau & Fleurence, 1993; Anfbal et al., 2014; Reticulata et al., 2002).

### **1.8.2 Gilthead seabream**

The family Sparidae, which includes seabreams and porgies, represents a diverse group of predominantly marine and brackish water fish with a global distribution across tropical, warm-temperate, and temperate oceans. Within this family, the gilthead seabream (*Sparus aurata*) emerges with a key role, holding both ecological significance and paramount importance in marine aquaculture (FAO, 2022). Gilthead seabream can be cultured through various methods, including extensive multitrophic or semi-intensive production in earth ponds, highly intensive land-based systems, as well as inshore and offshore sea cages (Basurco et al., 2011). This species demonstrates remarkable adaptability, thriving across a broad range of salinities and temperatures, provided they do not drop below 4 °C (Comi et al., 2022). Additionally, seabream showcases robust physiological mechanisms to maintain stable acid/base balance, even when confronted with varying ambient pH levels (optimal within the range of 6.5 to 8.5), and its capacity to modulate responses to dissimilar dissolved oxygen concentrations contributes to its consistent performance (Algers et al., 2008). It has been widely employed in various research studies, exemplified by Ashkenazi et al. (2019) who explored the integration of seabream with three marine macroalgae (*Ulva rigida*, *Gracilaria conferta*, *Hypnea musciformis*).

## **1.9 Objectives**

This study aims to understand the ability of *Ulva* sp. to bioremediate dissolved nutrients in a recirculating aquaculture system. The mechanical filtration system does not effectively regulate nutrient levels, productivity, or biological security. Therefore, it is

crucial to investigate how the *Ulva* sp. system responds to nutrient intake. So, one of the objectives of this work is to compare the results, in terms of water quality, fish performance, and *Ulva* sp. productivity integrated in RAS (IRAS) against a RAS without macroalgae.

Is also one of the purposes of the study to investigate how different densities can influence the productivity and growth rate of macroalgae. By investigating *Ulva* sp. as a biofiltration unit, it is expected to increase the knowledge of using this genus and the financial potential of growing commercially valuable seaweed species in closed systems.

## 2. Material and Methods

The trial took place for a period of five months. From the 25<sup>th</sup> of January to the 25<sup>th</sup> of April, 2023, the bacterial biofilter underwent maturation (section 2.2). Following that, the IRAS vs RAS trial started and lasted for two months, finishing on the 23<sup>rd</sup> of June. This work took place at Estação Piloto de Piscicultura de Olhão (EPPO, Portugal). The gilthead seabream, *Sparus aurata*, was the fish species used in this trial, while the macroalgae was *Ulva* sp., at three different densities.

### 2.1 Cultivation systems

The independent cultivation systems, IRAS and RAS, were placed outdoors and are represented in Figure 2.1. Each system comprised two tanks of 3000 L with fish; nine tanks of 200 L, with or without macroalgae, for IRAS and RAS respectively; and one tank of 1700 L functioning as the bacterial and mechanical filtration unit (FA and FB). The 1700 L tank filtration unit had a mechanical filter to retain the bigger particles, a skimmer (C) to remove small dimension particles from the system, oyster shells for pH buffering and a bacterial biofilter. The injection of pure oxygen into the water outlet was done in this tank, which had also a refrigerator to cool the water when the temperatures were too high (above 24 °C).

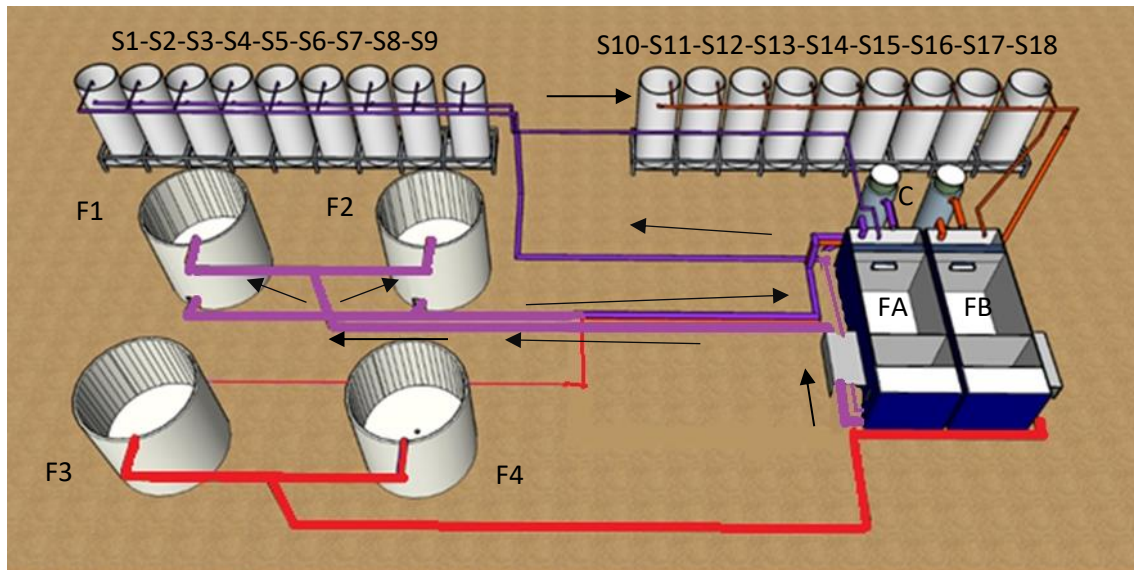


Figure 2.1 Schematic view from above of the experimental IRAS and RAS. S1 to S18 represent the 200 L tanks. F1 to F4 represent the fish tanks (3000 L). FA and FB: filters. C: skimmers (tubes with red color represent the IRAS system and tubes with purple color the RAS system).

Filtration unit A received the water from the fish tanks, F1 and F2. This water went through the filters and the skimmer (purple tubes) and some parts returned to fish tanks (F1, F2) and other to tanks S1-S9, containing only seawater. From the tanks S1-S9, the water returned to the filtration unit A and then the process restarts. Tanks F3 and F4 released the water to the filtration unit B and skimmer, and then some parts went directly to the fish tanks and others to the macroalgae tanks S10-S18. From these tanks, the water returned to the filtration unit B and the process restarts (red tubes). The water renovation was established at 10% of the total volume of the system at the beginning of the trial and increased to 20% on the 3<sup>rd</sup> day due to an increase in ammonia and nitrite levels. From this day until the end of the experiment, the water renovation was established at 20%. Daily measurements in the water flow rate were made in the fish tanks, 200 L tanks, and filter outflow, with the help of a beaker, to guarantee the same conditions in the entire trial, preventing overflow and reaching the maximum capacity in the system. The flow rate was calculated according to the maximum capacity of the system, the capacity to work without overload, and to reach the maximum of recirculation.

## **2.2 Bacterial biofilter preparation**

The bacterial biofilter was matured and then transferred to the RAS and IRAS filtration units before the beginning of the experiment. To clean the bacterial biofilter, they were placed in a square fiberglass tank to be washed and to remove the particles attached to them. To proceed with the maturation process, they were moved to a 3 m<sup>3</sup> tank with seawater. The plastic surface for bacterial growth was seeded with Nitribiotic (TMC, UK) which was a combination of probiotic and nitrifying bacteria in one preparation. This allowed the nitrifying bacteria to efficiently initiate the nitrification process, following the manufacturer's instructions for inoculation. Ammonium chloride was added to maintain an ammonia concentration of 3 to 5 mg L<sup>-1</sup> in the water, adjustments were made based on changes in ideal NH<sub>3</sub> levels. According to some authors, pH values of 6.8-7.8 are considered ideal for nitrifying bacterial development (Antoniou et al., 1990; Jones & Paskins, 1982), thus pH was maintained between those values in the experiment to assure ideal conditions. Alkalinity values were maintained around 150 mg CaCO<sub>3</sub> L<sup>-1</sup> for Nitrosomonas and 200-250 mg CaCO<sub>3</sub> L<sup>-1</sup> for Nitrobacter, which were adjusted using sodium bicarbonate as required. The TMC kit for Alkalinity/KW, was used. Strong aeration was provided, NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub> and NO<sub>3</sub> levels were regularly measured using TMC kits. Once the maturation had been verified and the ammonia and

nitrite values had been used efficiently by the bacteria throughout the day, the biofilter were transferred to the system and the trial has started.

### **2.3 Initial biomasses – Fish and Macroalgae**

The fish used was juvenile gilthead seabream originated from EPPO-IPMA (Estação Piloto de Piscicultura de Olhão - Instituto Português do Mar e Atmosfera) broodstock reared and maintained in the facilities until the beginning of the experiment. The two systems had a total of four seabream tanks starting at the same density of 12 kg m<sup>-3</sup>. All four tanks were under the same conditions of aeration and water flow rate, the flow rate was established at 2000 mL sec<sup>-1</sup> and was daily measured to ensure no variation between the fish tanks from RAS and IRAS treatments. The fish were hand-fed with a commercial diet (standard subtle orange, 4 mm, Aquasoja, Portugal) four times a day, at 9h30, 12h30, 14h30, and 16h30. The feed ratio was increased daily according to the manufacturer feeding table, assuming a potential fish growth of 0.8% day<sup>-1</sup>.

*Ulva* sp. was provided from previous experiments in EPPO - IPMA. The *Ulva* sp. was placed according to Figure 2.1 in tanks S10 – S18 belonging to the IRAS treatment. In this system, macroalgae were introduced at three different densities, 1.0, 1.5, and 2.0 kg m<sup>-2</sup>, in triplicate, with an initial stocking biomass of 229, 344, and 458 g, respectively. All the 200 L tanks were equipped with strong aeration and filters (with a net mesh small enough to not let the macroalgae cross to the rest of the system), flow rate was 111 mL sec<sup>-1</sup>, which was measured every day to maintain the same conditions between the two treatments.

### **2.4 Monitoring and sampling**

#### **2.4.1 Water parameters**

Water parameters such as dissolved oxygen, temperature, and pH were measured every morning around 11 am. Temperature and dissolved oxygen were measured with a Sinergia Oximeter and pH with an Accumet AB150 pH meter. These parameters were measured from the four fish tanks (F1, F2, F3, and F4), filtration units (FA, FB), and three 200 L tanks in each system (S1, S3, S6 and S14, S15, and S16), in the morning. Salinity was measured at 4 pm every day using a refractometer.

To monitor the water quality and bacterial biofilter performance, ammonia and nitrite quick tests (TMC kits) were used every morning in one fish tank from each system (F1 and F3), before feeding time, following the instructions from the manufacturer.

Water samples for further nutrient analyses were also taken three times a week (Monday, Wednesday, and Friday) at 10h00 from one fish tank in each system (F1 and F3), one 200 L tank in each system (S3 and S14), and from filtration units (FA and FB). In the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> weeks of the trial, samples were also taken, on Wednesday, every two hours, during light time, from 6h00 to 22h00. The water was sampled from the opposite side of the flowthrough in the fish and 200 L tanks and in the filtration units it was sampled in the compartment of the bacterial biofilter and mechanical filter.

After the water was sampled from the tanks, it was filtered (membrane cellulose acetate filter, 0.45  $\mu\text{m}$ ) to duplicated and labeled vials from each tank. The material used was washed and dried to be placed in acid until the next water sampling. After the vials were filled, they were placed in the freezer and kept until analysis of  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations. The nutrients were analyzed in an analytical laboratory by a Segmented Flow Analyzer (SFA) in MARINOVA company.

#### **2.4.2 Macroalgae biomass**

The sampling of macroalgae biomass was conducted once a week for the duration of the trial (Figure 2.2). The macroalgae biomass from each tank was removed and weighed. To obtain accurate weight measurements, it was necessary to drain the water from the 200 L tanks (Figure 2.2 A), remove the macroalgae, and to take all the excess of water. This was achieved by using a small net to transfer the algae and applying pressure to drain the water. The tanks, aeration systems, and filters were cleaned and replaced during this process. When the macroalgae biomass was weighed, samples of algae at the initial density were randomly placed in the tanks, to ensure that the same density was not always placed in the same tank between weeks (Figure 2.2 B).



Figure 2.2 Overview of *Ulva* sp. sampling. A = Beginning of the draining process of *Ulva* sp. from the 200 L tanks. B = *Ulva* sp. separated by densities ready to be replaced in the tanks.

Additionally, a subsample of excess biomass ranging between 10 and 30 g was taken from each tank. This subsample was then dried at 60 °C for ~120 h. After drying, the dry weight of the macroalgae was determined, and fresh weight: dry weight (FW: DW) was calculated. The dried macroalgae were ground into a fine powder using a mortar and pestle for posterior N analyses. The ground sample was stored in Eppendorf tubes inside a desiccator until analysis. The analyses were performed by Organic Elemental Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS) in MARINOVA facilities.

Specific growth rate (SGR) and daily yield were calculated to assess the growth of *Ulva* sp. at different densities. N yield was calculated to compare the N removal of the macroalgae at different densities.

$$\text{SGR (\% d}^{-1}\text{): } ((W_t \times W_i^{-1})^{(1/t)} - 1) \times 100$$

$$\text{Daily yield (g m}^{-2} \text{ d}^{-1}\text{): } ((W_t - W_i) \times A^{-1}) \times t^{-1}$$

$$\text{N yield (g N m}^{-2} \text{ d}^{-1}\text{)} = Y \times N_c$$

$W_i$  = initial biomass (g),  $W_t$  = final biomass (g),  $t$  = total of culture days,  $A$  = area of surface cultivation tank ( $\text{m}^2$ ),  $Y$  = biomass yield ( $\text{g DW m}^{-2} \text{ d}^{-1}$ ),  $N_c$  = nitrogen content (%).

### 2.4.3 Fish biomass

Fish sampling was conducted at the start and end of the trial. At the beginning of the trial, the fish were collected from the EPPO facilities, and at the end of the trial, they were collected from the IRAS and RAS tanks. The fish were then transferred to a small tank containing 0.5 mL L<sup>-1</sup> of anaesthesia (2-phenoxyethanol, 99% from PanReac AppliChem, Portugal). Total length was measured from the first 40 fish from each tank and all the fish were weighed, using a ruler and a scale, respectively.

Fish growth performance was analyzed using the thermal-unit growth coefficient (TGC), weight gain (WG), and condition factor (K), which were calculated with the following formulas:

$$\text{TGC} = ((W_f^{1/3} - W_i^{1/3}) \times \sum DD^{-1}) \times 100$$

$$\text{WG} = ((W_f - W_i) \times W_i^{-1}) \times 100$$

$$\text{K} = (W_f \times L^{-3}) \times 100$$

$W_i$  = initial weight (g),  $W_f$  = final weight (g),  $DD$  = degree days (°C),  $L$  = length.

#### 2.4.3.1 Hematological parameters

At the end of the trial, blood was collected from 10 fish per tank (20 per treatment) for hematocrit quantification and hemoglobin determination. The fish were euthanized with a knife for this analysis. Hematocrit was determined using microhematocrit tubes (capillary tubes plain, 75 mm; Super Rior, West Germany), which were centrifuged at 5,000 g for 5 min in a Hettich EBA 21 centrifuge (Tuttlingen, Germany). Hemoglobin was determined with a commercial kit for clinic application (QCA, Spain).

#### 2.4.3.2 Gill histology

At the end of the trial 10 fish per tank (20 per treatment) were euthanized with a knife, gills were collected and fixed in 4% buffered formaldehyde for 24 h. Subsequently, the tissues were washed three times for 15 min each with 0.1 mM phosphate saline buffer (PBS) at pH 7.4, followed by tap water, and then stored in 70% ethanol until paraffin embedding. When necessary, tissues underwent decalcification using an acid solution of 8% HCl and 8% formic acid (1:1) for 12 hours before embedding. Thin sections

measuring 6–7  $\mu\text{m}$  were prepared, stained with hematoxylin-eosin, and examined under a binocular microscope. Gill sections were evaluated by the observation of two primary lamellae and their associated secondary lamellae using a histopathology scoring system, which was based on previous scoring systems used for assessing histological changes in fish gills (Araújo-Luna et al., 2018; Baxter et al., 2011; Bosch-Belmar et al., 2016). The scoring system focused on three main pathologies: epithelial hyperplasia (increased cell production), lamellar fusion, and cellular anomalies (degeneration and necrosis). Each of these pathologies was assigned a primary score ranging from 0 to 3, depending on their presence, extent, and severity.

Additionally, secondary parameters were evaluated, and each of them received a score of either 0 or 1, indicating the absence or presence of specific conditions. These secondary parameters included hypertrophy (enlargement of cells), oedema (accumulation of fluid), eosinophilic granular cells, inflammation, circulatory damage, and congestion of secondary lamellae.

The total histopathology score for each gill section was calculated by adding up the primary scores and the scores for the secondary parameters. The resulting total score allowed categorizing the severity of the gill pathology as:

- 0 to 3: No significant pathology.
- 4 to 6: Mild gill pathology of minor clinical significance.
- 7 to 9: Moderate gill pathology of clinical significance.
- 10: Severe gill pathology of high clinical significance.

This histopathology scoring system provided a comprehensive assessment of the gill health in the fish and helped to identify the level of pathological changes present, which could have clinical implications for the overall health and performance of the fish.

## **2.5 Data analysis**

The results were analyzed using the R software package version 4.3.1. Results expressed as percentages were transformed (arcsine square root) before statistical analysis (Ennos, 2007). Data was given as mean  $\pm$  standard deviation (SD). The null hypothesis was rejected at a significance level of 5%. Normality and homogeneity of data were tested beforehand using the Shapiro-Wilk and Levene's tests. When the assumptions were met, two-way ANOVA, one-way ANOVA, and independent t-tests were used to compare the

means of the data (two or more samples). When differences or interactions were found, Tukey's HSD test was performed to compare the means.

Two-way ANOVA was used to analyze nutrients, *Ulva* sp. SGR and Yield at different treatments and weeks/hours. One-way ANOVA was used to identify possible differences in *Ulva* sp. N content between treatments. The data from the fish were analyzed using independent *t*-tests.

When assumptions were not met, logarithmic or square root transformations were performed to normalize the data. If the logarithmic and square root transformations did not meet the requisites, Wilcoxon (two samples) was then used to compare the medians of the data or Mann-Whitney tests for data from fish.

### 3. Results

#### 3.1 Mechanical filtration

The results observed in the mechanic filtration unit (Figure 2.1) revealed an accumulation of particles on the mesh of the mechanical filters in both systems (A - RAS and B - IRAS). In the last weeks of the trial, cleaning the filters daily was necessary due to excessive accumulation in its central region (C). Cleaning the filters was difficult, due to the structure that they have to retain the organic matter, which resulted in an overflow in the last weeks of the trial. Notably, while each filter mesh contained a high volume of particles, only filter B experienced overflow on several days towards the end of the trial.

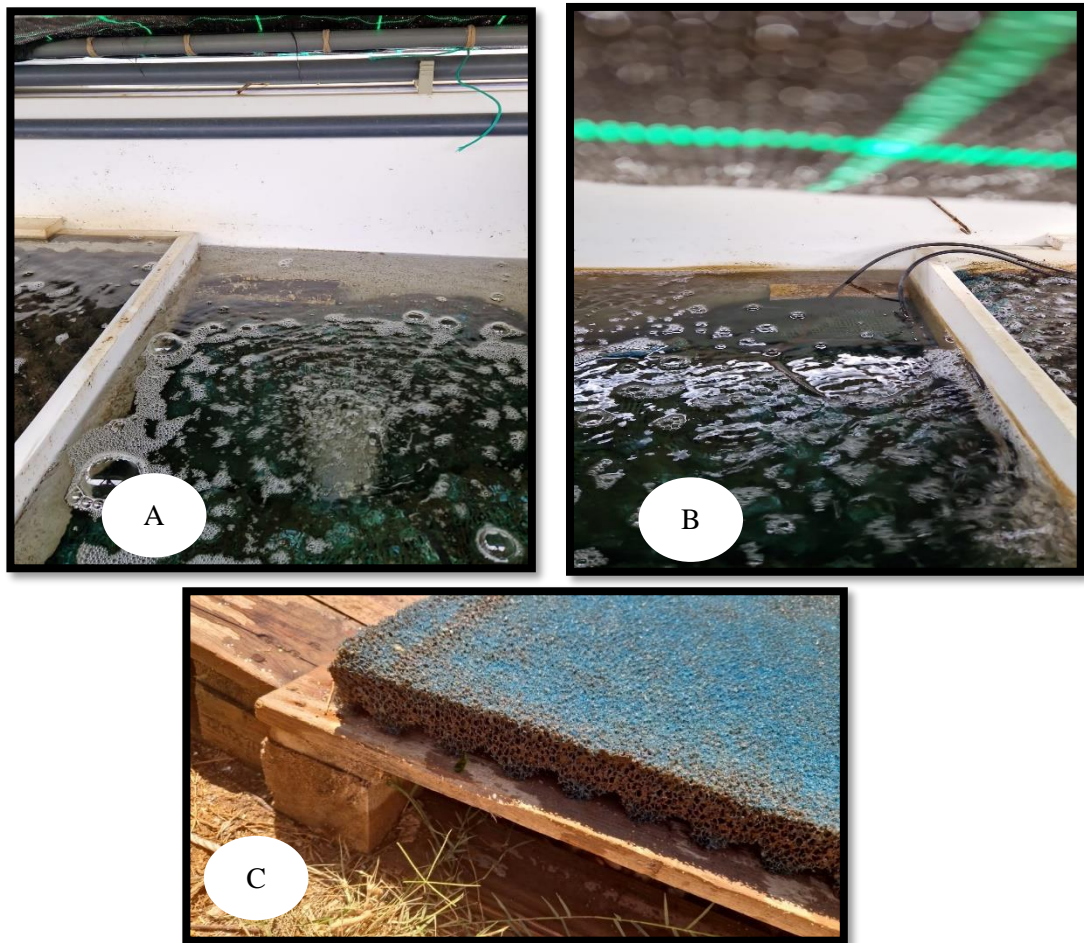


Figure 3.1 Accumulation of organic matter in the mechanical filters. Mechanical filter condition in the last weeks of trial. (A) RAS; (B) IRAS; (C) organic matter compacting in the filter.

### 3.2 Water parameters

The water parameters, temperature, dissolved oxygen, and pH had similar patterns between the two systems in the morning. The maximum temperature was reached in the last week of the trial in both systems (IRAS:  $24.3 \pm 0.89$  °C; RAS:  $24.2 \pm 0.87$  °C) and the lowest temperature in week four (IRAS:  $22.7 \pm 0.42$  °C and RAS:  $22.8 \pm 0.47$  °C) (Figure 3.2).

For oxygen (Figure 3.3), the higher values were registered in week two (IRAS:  $7.8 \pm 1.67$  mg L<sup>-1</sup> and RAS:  $7.5 \pm 1.28$  mg L<sup>-1</sup>) while the lower O<sub>2</sub> concentrations were registered for IRAS in week one ( $6.9 \pm 1.58$  mg L<sup>-1</sup>) and for RAS in week eight ( $6.3 \pm 1.25$  mg L<sup>-1</sup>).

The pH was also apparently similar among RAS and IRAS treatments (Figure 3.4), the maximum values were in week two (IRAS:  $7.8 \pm 0.21$  and RAS:  $7.6 \pm 0.18$ ) and the minimum values were verified in week seven (IRAS:  $7.4 \pm 0.17$  and RAS:  $7.3 \pm 0.09$ ).

The mean salinity during the trial was  $36.4 \pm 0.82$  for the RAS system and  $36.5 \pm 0.78$  for the IRAS system, similar between the two systems.

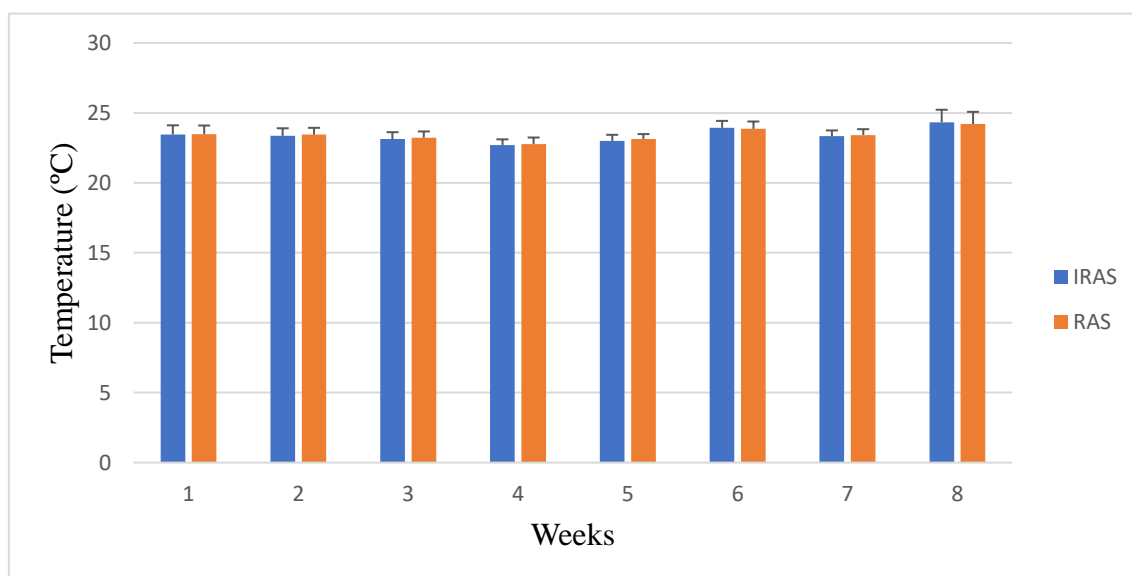


Figure 3.2 Temperature in the IRAS and RAS in the morning during the eight-week trial. Data are expressed as mean  $\pm$  SD.

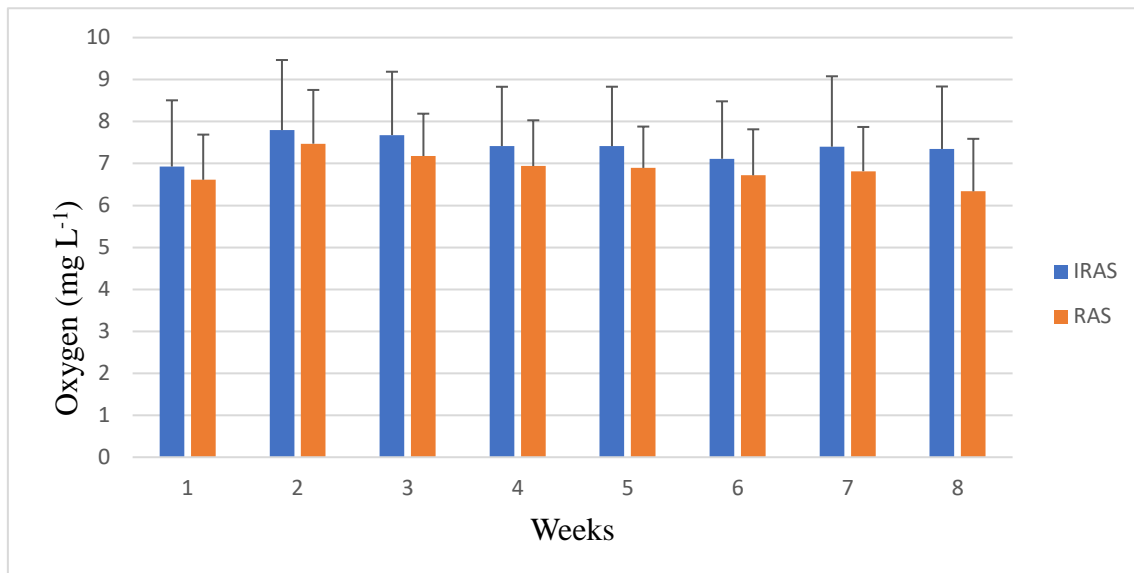


Figure 3.3 Dissolved oxygen values in the IRAS and IRAS in the morning during the eight-week trial. Data are expressed as mean  $\pm$  SD.

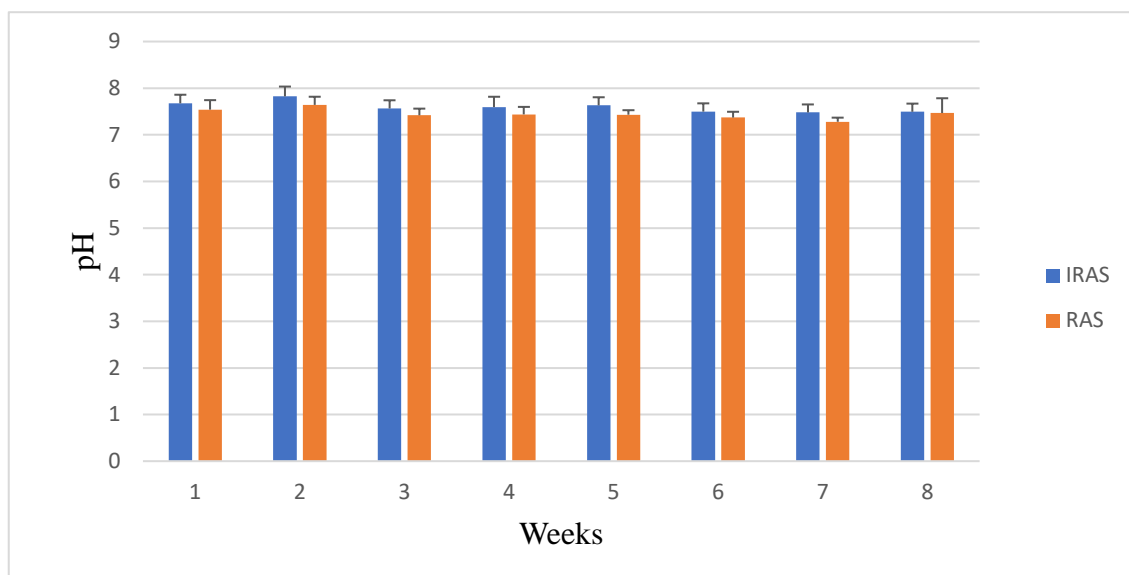


Figure 3.4 pH values in the IRAS and RAS in the morning during the eight-week trial. Data expressed are as mean  $\pm$  SD.

### 3.3 Dissolved nutrients

#### 3.3.1 Nutrient monitorization along the trial

Possible differences between parts of the systems (IRAS and RAS) were analyzed among the nutrients.  $\text{NH}_4$  showed significant differences in different parts of both systems. Higher values were found in the fish tanks (IRAS:  $1.1 \pm 0.38$  and RAS:  $1.1 \pm 0.36 \text{ mg L}^{-1}$ ) when compared with the filters and 200 L tanks of the same system (IRAS: filters  $0.6 \pm 0.26$ ; tanks without seaweed  $0.6 \pm 0.33$  and RAS: filters  $0.7 \pm 0.31$ ; tanks with seaweed  $0.7 \pm 0.29 \text{ mg L}^{-1}$ ) ( $p < 0.05$ ). No differences in parts of both systems were found for  $\text{PO}_4$ ,  $\text{NO}_2$  and  $\text{NO}_3$  ( $p > 0.05$ ). No significant interaction was found in all

nutrients between treatments and parts of the system ( $p > 0.05$ ). Upon analyzing the results by systems and weeks, no differences were found between the two systems in all nutrients ( $p > 0.05$ ), but significant differences were observed among the nutrients over the weeks. The  $\text{NH}_4$  values were significantly higher in weeks six and seven compared to all other weeks in the trial ( $p < 0.05$ ), as seen in Figure 3.5. The  $\text{PO}_4$  results showed significantly lower values in weeks one and five when compared to all the other weeks ( $p < 0.05$ ), as shown in Figure 3.6.  $\text{NO}_2$  showed significantly higher values at the beginning of the trial. Weeks one, two, and three were significantly higher than the other weeks ( $p < 0.05$ ), as indicated in Figure 3.7. Regarding  $\text{NO}_3$ , week one had significantly lower values than all other weeks, while weeks two, three, and five also had significantly lower values than weeks four, six, and seven ( $p < 0.05$ ), as shown in Figure 3.8.

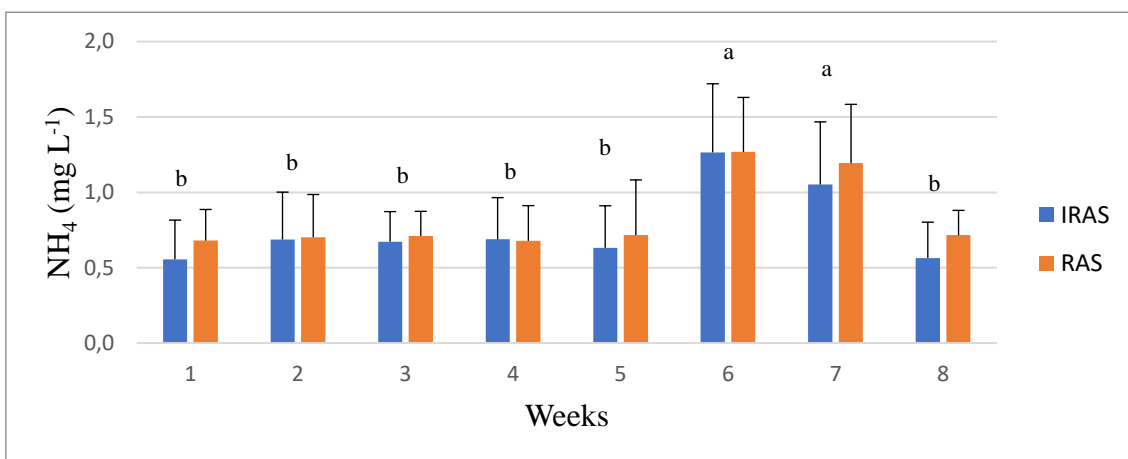


Figure 3.5  $\text{NH}_4$  concentration in the IRAS and RAS during the eight-week trial. Different lowercase letters represent significant differences ( $p < 0.05$ ) between weeks. Absence of uppercase letters represent no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 9$ ).

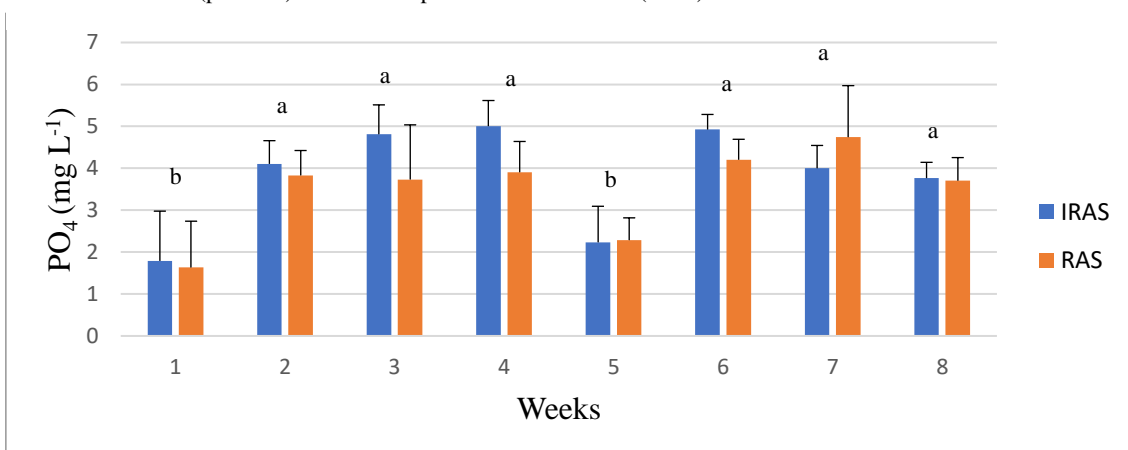


Figure 3.6  $\text{PO}_4$  concentration in the IRAS and RAS during the eight-week trial. Different lowercase letters represent significant differences ( $p < 0.05$ ) between weeks. Absence of uppercase letters represent no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 9$ ).

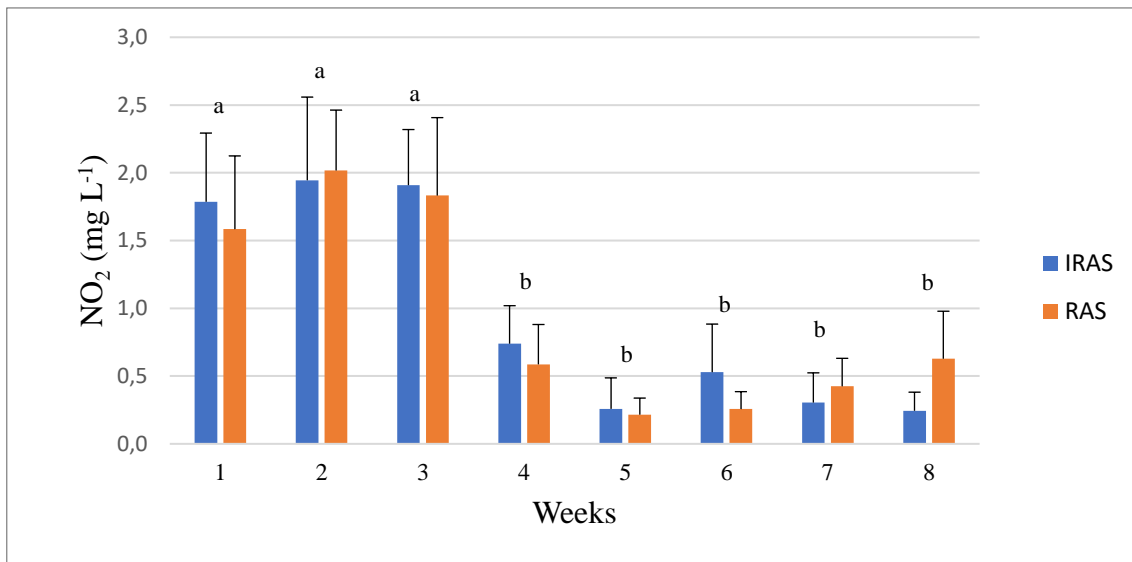


Figure 3.7 NO<sub>2</sub> concentration in the IRAS and RAS during the eight-week trial. Different lowercase letters represent significant differences ( $p < 0.05$ ) between weeks. Absence of uppercase letters represent no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 9$ ).

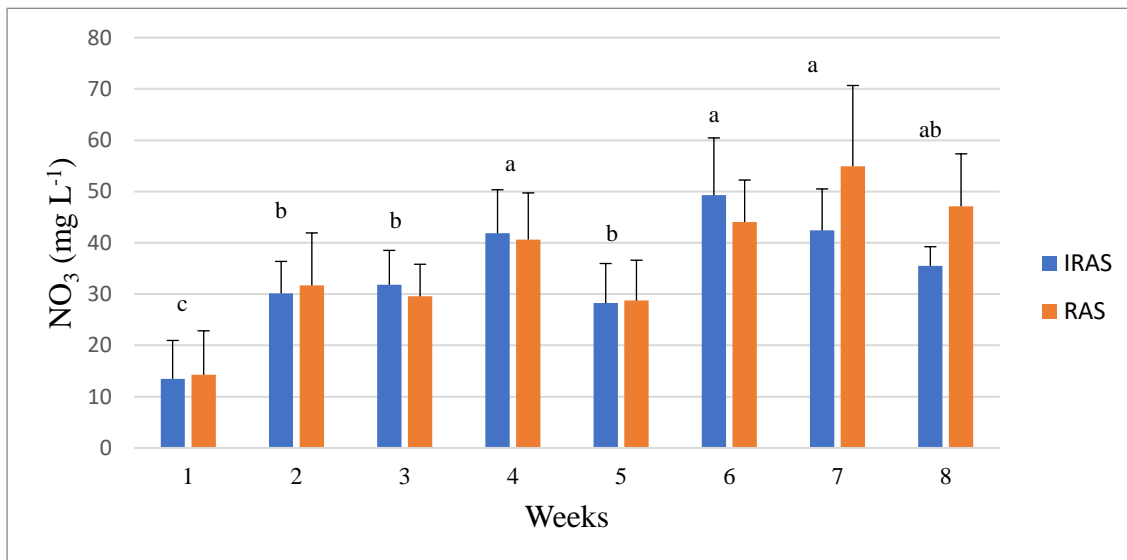


Figure 3.8 NO<sub>3</sub> concentration in the IRAS and RAS during the eight-week trial. Different lowercase letters represent significant differences ( $p < 0.05$ ) between weeks. Absence of uppercase letters represent no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 9$ ).

### 3.3.2 Nutrient analysis during a daylight cycle

The NH<sub>4</sub> results showed no significant differences between the IRAS and RAS during the daylight cycle. However, the presence of this nutrient in the systems was influenced by the time of day. Values registered at 20h00 and 22h00 were significantly higher than values registered at 08h00, 10h00, 12h00, and 14h00 ( $p < 0.05$ ) (Figure 3.9). On the other hand, there were differences in PO<sub>4</sub> concentrations between the two systems, but no significant changes were found along the time of day. This nutrient was significantly higher in the IRAS treatment compared to the RAS treatment, with the

exception of 18h00 hours, when it was higher in the RAS than in the IRAS ( $p < 0.05$ ) (Figure 3.10). Similarly,  $\text{NO}_2$  behaved in the same way with no significant differences found between sampling hours, but significantly higher values were verified in IRAS compared to the RAS system ( $p < 0.05$ ) (Figure 3.11). Finally,  $\text{NO}_3$  in the systems did not differ either between treatments or sampling hours ( $p > 0.05$ ) (Figure 3.12).

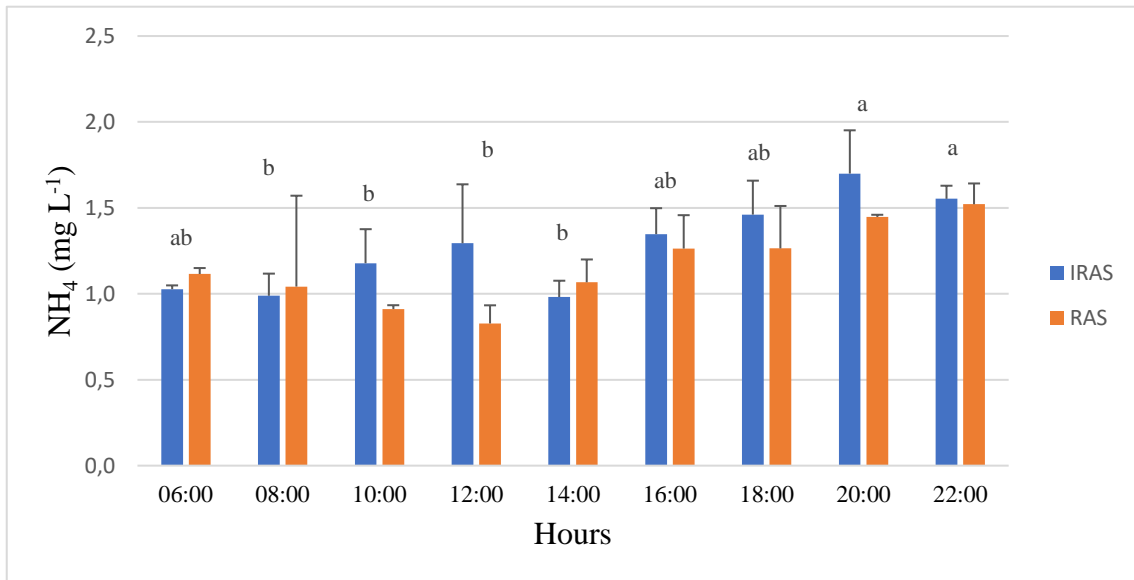


Figure 3.9  $\text{NH}_4$  concentrations along a daylight cycle in fish tanks from IRAS and RAS. Different lowercase letters represent significant differences ( $p < 0.05$ ) between sampling times. Absence of uppercase letters represent no significant differences between systems ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

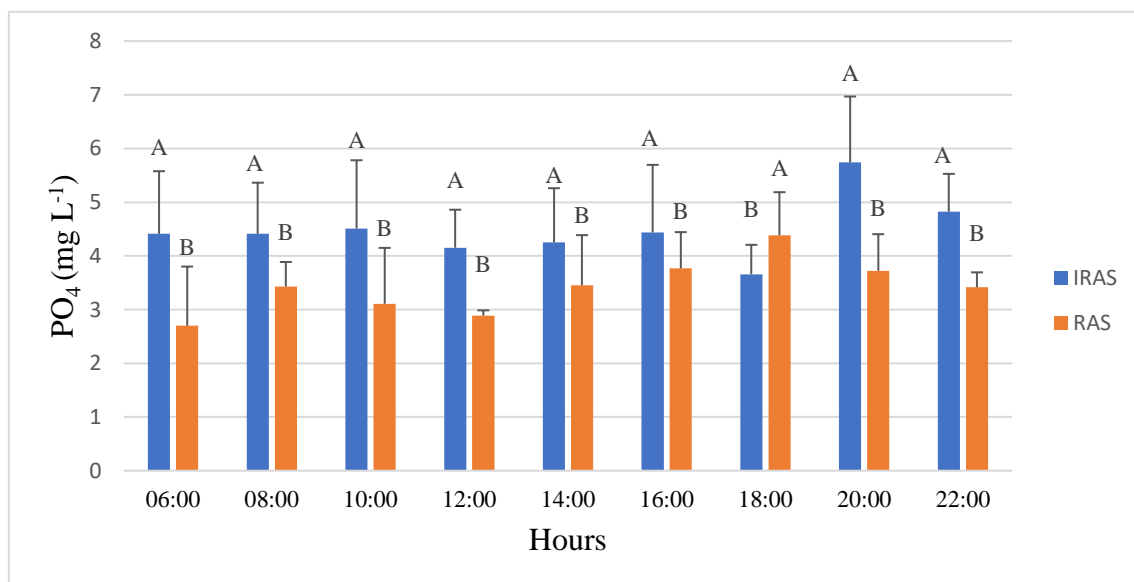


Figure 3.10  $\text{PO}_4$  concentrations along a daylight cycle in fish tanks from IRAS and RAS. Different uppercase letters represent significant differences ( $p < 0.05$ ) between the two systems. Absence of lowercase letters represent no significant differences between hours ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

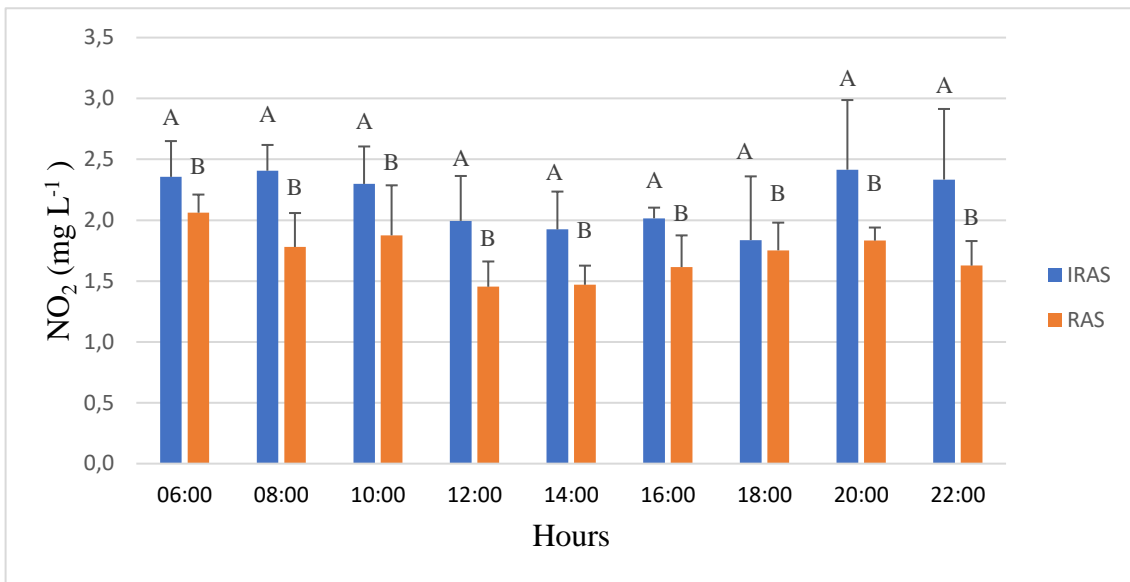


Figure 3.11 NO<sub>2</sub> concentrations along a daylight cycle in fish tanks from IRAS and RAS. Different uppercase letters represent significant differences ( $p < 0.05$ ) between systems. Absence of lowercase letters represent no significant differences between hours ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

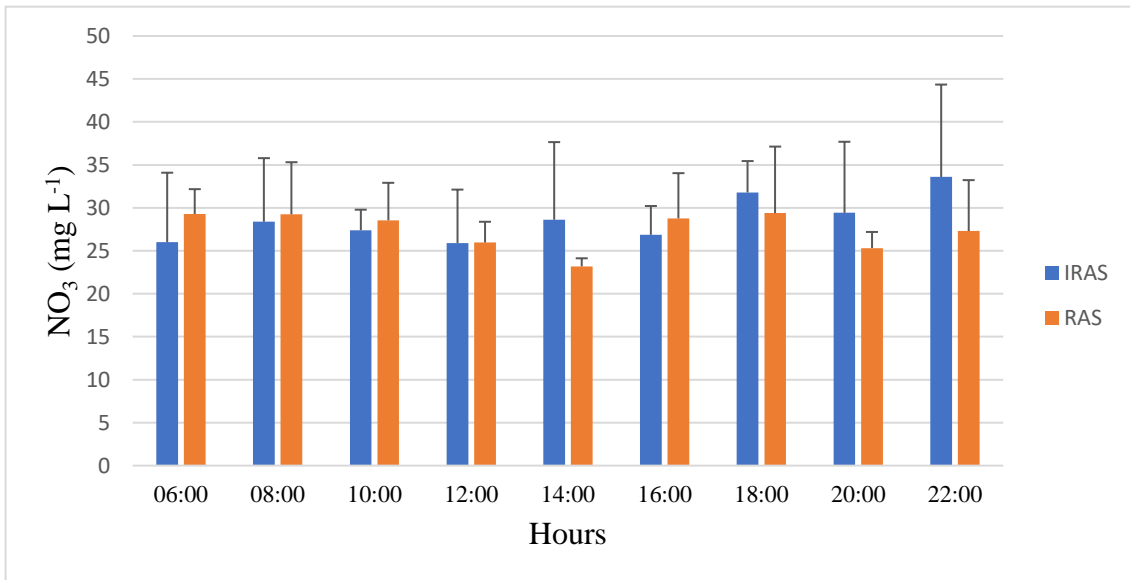


Figure 3.12 NO<sub>3</sub> concentrations along a daylight cycle in fish tanks from IRAS and RAS. The absence of letters represents no significant differences ( $p > 0.05$ ) between sampling times or systems. Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

### 3.4 *Ulva* sp.

#### 3.4.1 Growth and yield

SGR and yield of *Ulva* sp. in IRAS were compared between densities and weeks of harvest.

In the trial, the SGR of density 1.0 Kg FW m<sup>-2</sup> was significantly higher than densities 1.5 and 2.0 kg FW m<sup>-2</sup> ( $p < 0.05$ ). Similarly, the SGR of density 1.5 Kg FW m<sup>-2</sup> was significantly higher than that of density 2.0 kg FW m<sup>-2</sup> ( $p < 0.05$ ). The SGR values

for the density 1.0 kg FW m<sup>-2</sup> varied from 31.1 ± 1.63% d<sup>-1</sup> to 35.5 ± 1.17% d<sup>-1</sup>. For the density of 1.5 Kg FW m<sup>-2</sup>, the SGR varied from 22.1 ± 1.38 % d<sup>-1</sup> to 29.0 ± 1.05% d<sup>-1</sup>. The density of 2.0 kg FW m<sup>-2</sup> showed the lowest SGR values across all harvests, varying from 19.4 ± 1.27% d<sup>-1</sup> to 24.7 ± 0.98% d<sup>-1</sup>. The significant differences in SGR values were found between the weeks of harvest. Specifically, the SGR value in week one was significantly lower than the SGR value in week three. Additionally, the SGR values in weeks six and eight were significantly lower than in all other weeks, except the first week of the trial (p < 0.05). No significant interaction between densities and weeks were found (p > 0.05) (Figure 3.13).

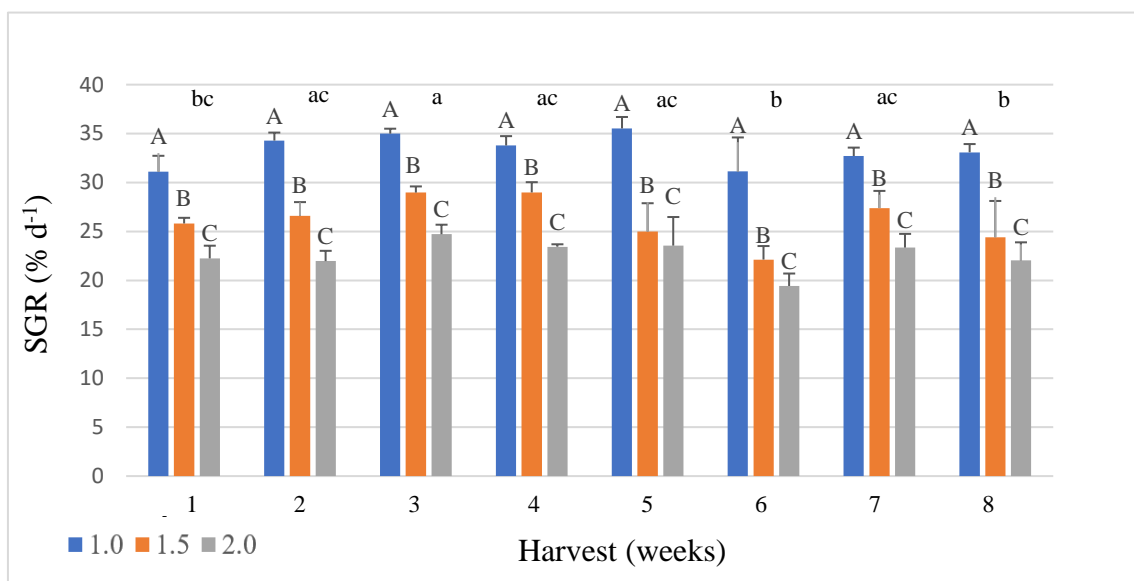


Figure 3.13 Specific growth rate of *Ulva* sp. by initial density (1.0, 1.5 and 2.0 kg m<sup>-2</sup>) and harvest weeks. Different uppercase letters represent significant differences (p < 0.05) between densities. Different lowercase letters represent significant differences between weeks (p < 0.05) Data are expressed as mean ± SD (n = 3).

In yield, no significant differences were found between densities (p > 0.05), but significant differences were found between the different weeks (Figure 3.14). Specifically, week one had significantly lower yield values compared to week three. Additionally, week six had significantly lower yield compared to all weeks, except for the first two weeks and the last week of harvesting (p < 0.05). No significant interaction was found between densities and weeks (p > 0.05).

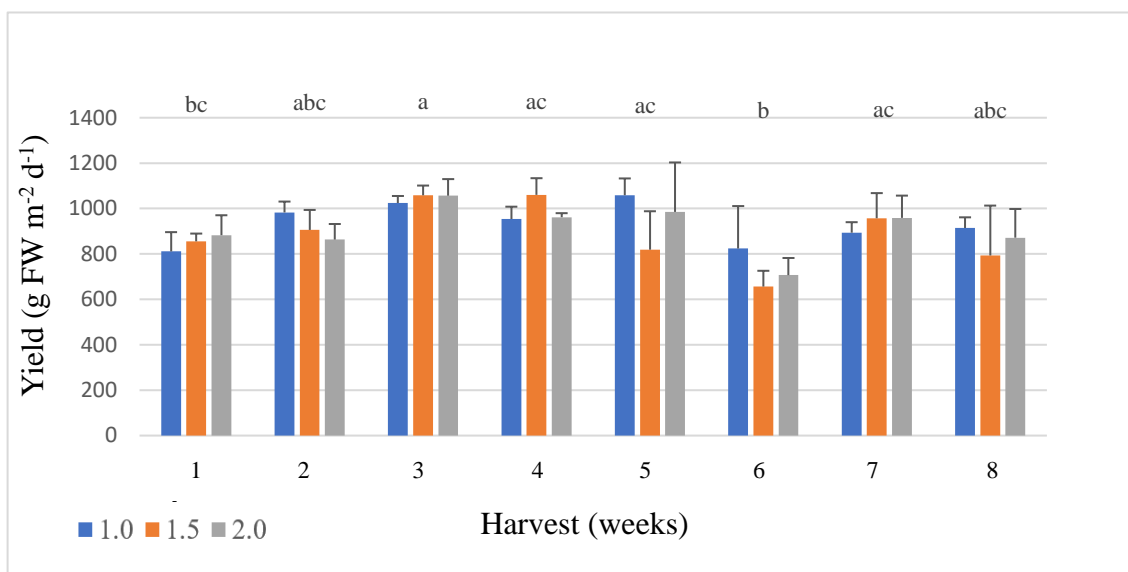


Figure 3.14 Yield of *Ulva* sp. by initial density and harvests (1.0, 1.5 and 2.0 kg m<sup>-2</sup>). Different lowercase letters represent significant differences ( $p < 0.05$ ) between the harvest weeks. Absence of uppercase letters represent no significant differences between densities ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

### 3.4.2 N removal

*Ulva* sp. N content was statistically analyzed between the biomass at week 8, at the three different densities, and the initial biomass. Results of *Ulva* sp. N content revealed no statistically significant differences among the three densities ( $p > 0.05$ ), but significantly higher values were found in the three densities when compared with the initial sample of *Ulva* sp. ( $p < 0.05$ ) (Figure 3.15). To better understand the results, week 8 N yield (g DW m<sup>-2</sup> d<sup>-1</sup>) was calculated and represented in Table 3.1, but no significant differences were found.

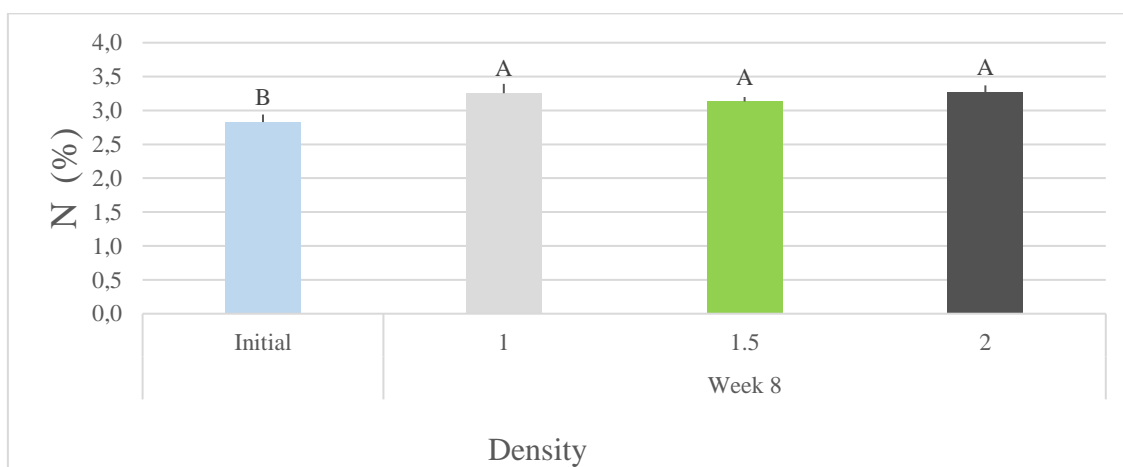


Figure 3.15 Nitrogen content (Nc) for the different densities of *Ulva* sp. at week 8 and from the initial sample. Different uppercase letters represent significant differences ( $p < 0.05$ ) between densities. Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

Table 3.1 *Ulva* sp. Week 8 N yield for each density (DW: dry weight). The absence of letters represents no significant differences between treatments. Data are expressed as mean  $\pm$  SD (n =3).

Harvest	Density (kg FW m <sup>-2</sup> )	N yield (g DW m <sup>-2</sup> d <sup>-1</sup> )
Week 8	1.0	4.4 $\pm$ 0.42
	1.5	4.1 $\pm$ 1.28
	2.0	3.7 $\pm$ 0.32

### 3.5 Fish performance

#### 3.5.1 Growth

No significant differences between treatments in terms of total size and weight were found at the outset of the experiment ( $p > 0.05$ ). Results at the end of the trial also revealed no significant difference between the IRAS and RAS treatments in terms of total length and weight ( $p > 0.05$ ) (Figures 3.16 and 3.17). The results indicate an increase in weight gain (WG) in fish from both systems and similar TGC values. K values were not statistically different between IRAS and RAS treatments ( $p > 0.05$ ) (Table 3.2).

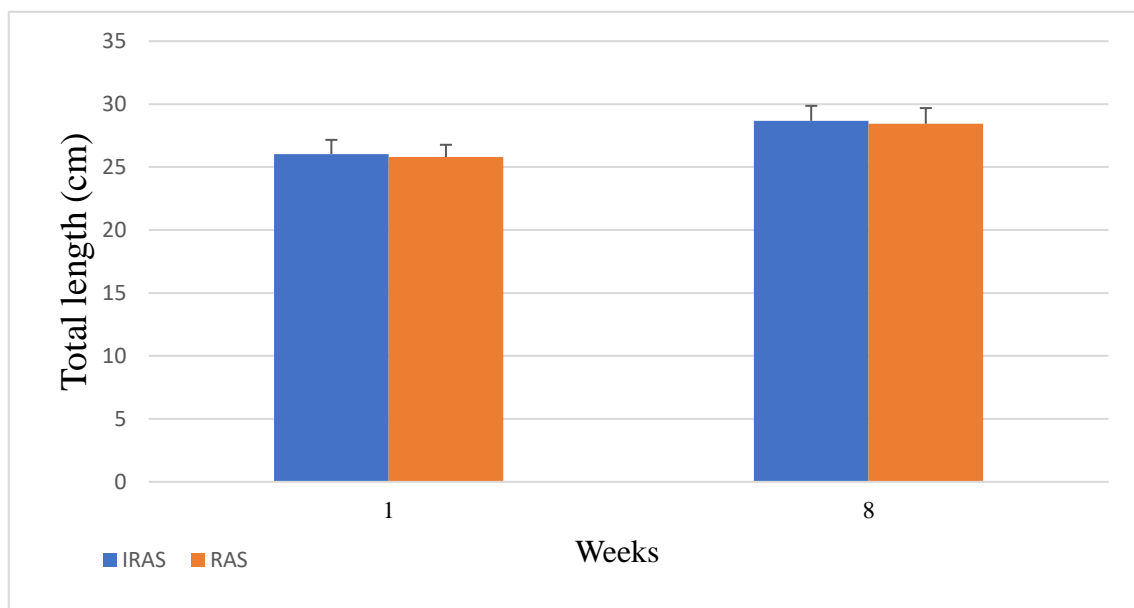


Figure 3.16 Fish length at the beginning and at the end of the trial in the two treatments. The absence of letters represents no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD (n = 40).

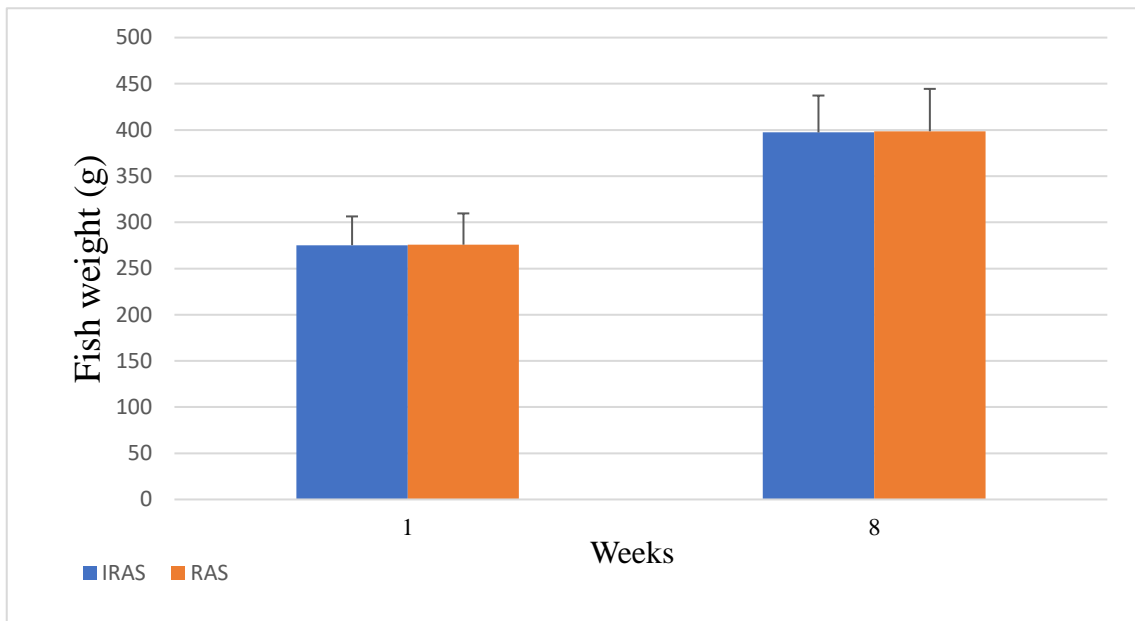


Figure 3.17 Fish weight at the beginning and at the end of the trial in the two treatments. The absence of letters represents no significant differences between treatments ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 260$ ).

Table 3.2 Thermal-unit growth coefficient (TGC), weight gain (WG) and condition factor (K) in gilthead seabream from IRAS and RAS treatments. The absence of letters represents no significant differences between treatments. Data are expressed as mean  $\pm$  SD (TGC and WG,  $n = 2$ ; K,  $n = 40$ ).

Treatment	TGC	WG (%)	K
IRAS	1.9 $\pm$ 0.41	41.6 $\pm$ 0.34	1.7 $\pm$ 0.10
RAS	2.4 $\pm$ 0.11	41.8 $\pm$ 0.33	1.8 $\pm$ 0.18

### 3.5.2 Hematological parameters

Values for hematocrit and hemoglobin were not significantly different ( $p > 0.05$ ) between the treatments. Hematocrit values ranged between  $45.50 \pm 2.45\%$  and  $48.00 \pm 4.87\%$  for IRAS and RAS treatments, respectively. For hemoglobin, values ranged from  $9.34 \pm 2.05$  to  $8.39 \pm 2.86$  g dL<sup>-1</sup> in the IRAS and RAS treatments, respectively (Figure 3.18).

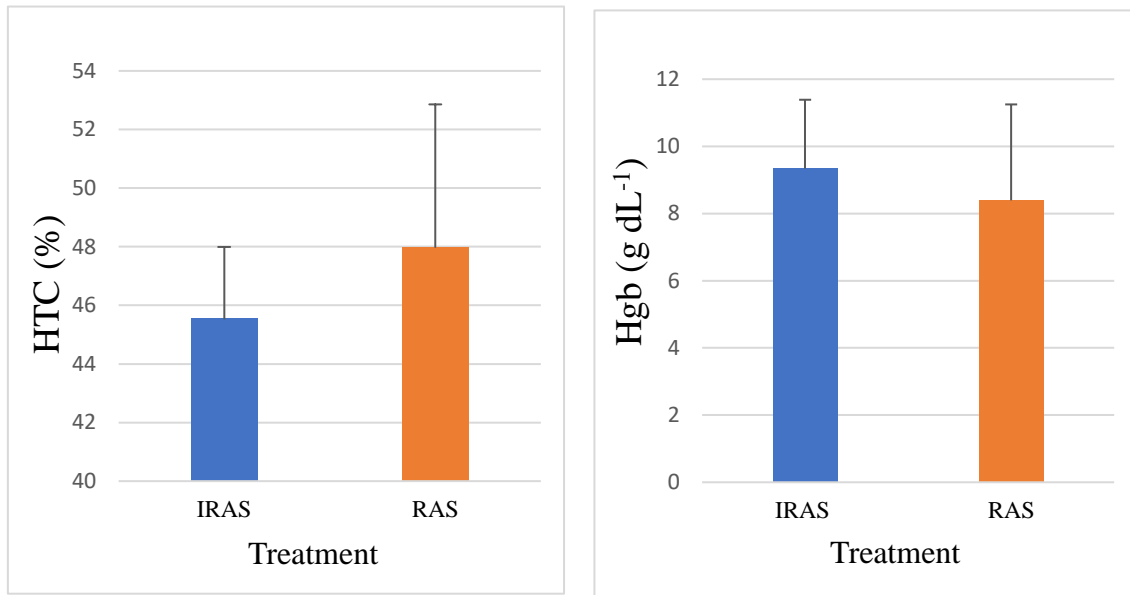


Figure 3.18 Hematocrit (HTC) and hemoglobin (Hgb) values in the two treatments. The absence of letters represents no significant differences ( $p > 0.05$ ) between treatments. Data are expressed as mean  $\pm$  SD ( $n = 20$ ).

### 3.5.3 Gill histology

Out of the 40 gills sampled, the majority exhibited favorable conditions for assessment. However, four gills, two from each treatment (IRAS, RAS), were deemed unsuitable for observation, likely due to distortion during the cutting and preparation process.

During the observation, the primary parameters most affected were epithelial hyperplasia and lamellar fusion, while prevalent issues in the secondary parameters included the presence of granular cells and congestion of secondary lamellae (Figure 3.19 A and B). Although other condition-related problems occasionally appeared, they were less common. One case of an extremely visible aneurysm in RAS and instances of degeneration/necrosis were observed in both treatments (Figure 3.19 C and D).

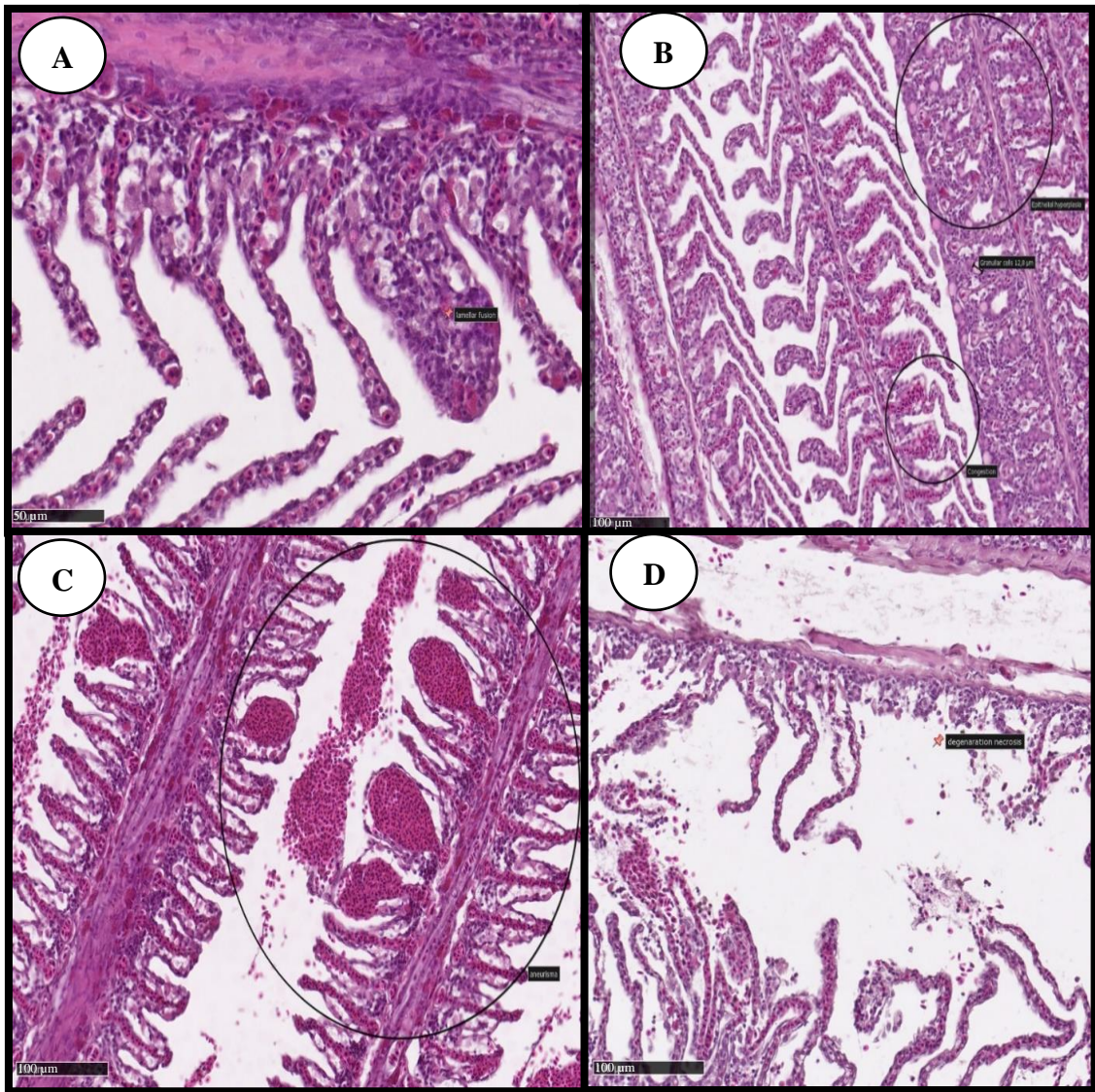


Figure 3.19 Histological cuts (H&E staining) on gills from both treatments. (A) Visible presence of lamellar fusion; (B) presence of granular cells, epithelial hyperplasia, and congestion of secondary lamellae; (C) visible aneurism; (D) degeneration/ necrosis.

Fish reared in both systems displayed some gills anomalies, but no significant differences were found between treatments ( $p > 0.05$ ). Scores results were 3.9 and 5.0 for fish from RAS and IRAS, respectively (Figure 3.20). According to the scoring system, the anomalies observed in the gills were classified as mild gill pathology of minor clinical significance.

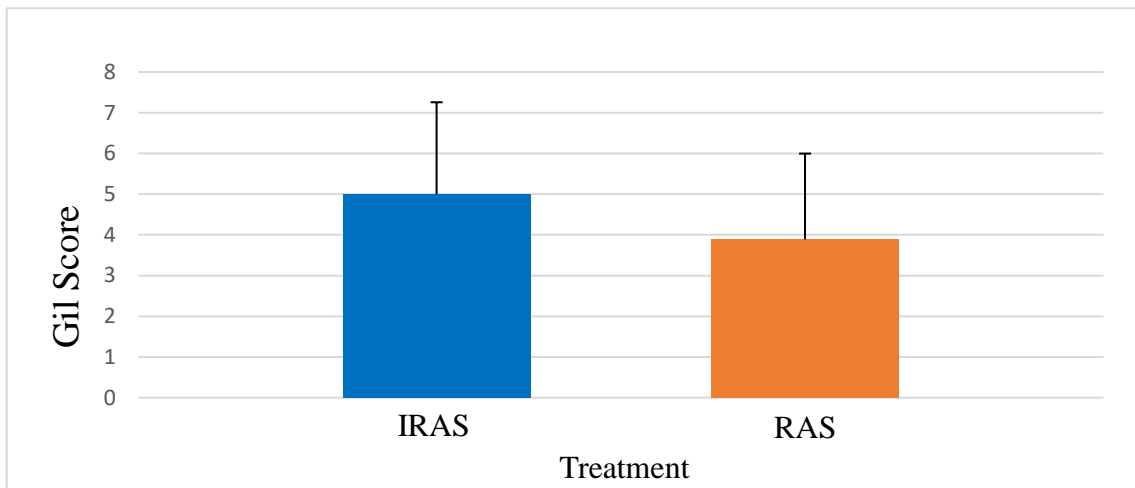


Figure 3.20 Semiquantitative analyses of gill damage in gilthead seabream from both treatments. The absence of letters indicates no significant differences. Data expressed as mean  $\pm$  SD (n =18).

#### 4. Discussion

Environmental parameters play a crucial role in the growth and health of aquatic organisms in RAS and IRAS. For the species studied in this experiment, *Ulva* sp. and gilthead seabream (*Sparus aurata*), maintaining optimal environmental conditions is crucial to ensure successful cultivation and system productivity. The water temperature remained between 22 and 25 °C in both systems, within the ideal range for the growth of both *Ulva* sp. and seabream. In the study by Favot et al. (2019), better growth results were observed for *Ulva* sp. cultivated in Ria Formosa during the summer with temperatures between 22 and 30 °C. According to Xiao et al. (2016), the optimal temperature range for *Ulva prolifera* is between 20-25 °C, and for seabream ideal temperatures should be between 20-24 °C (Feidantsis et al., 2009; Kir, 2020). Both RAS and IRAS were equipped with refrigerators to maintain the temperature below 24 °C. However, in the last week of the study, temperature values above this limit were recorded in both systems. These results suggest that, despite the control measures implemented, ambient temperature may have influenced the results. Water pH values remained within the tolerable range in both systems, which, when below 6.5 and above 9, can impair the growth and health of seabream (Algers et al., 2008). These results indicate that pH conditions were suitable for species development in both systems. Higher dissolved oxygen values were expected in the IRAS treatment due to the photosynthesis of the *Ulva* sp., however, this was impossible due to oxygen injections made in both treatments to keep them under similar conditions. Comparing oxygen results around 7 mg L<sup>-1</sup> with other studies, the results in this trial fall within values considered ideal; values below 7.4 mg L<sup>-1</sup> in fish are a stressful condition, which have been demonstrated to reduce growth rate, feeding activity and could also result in gill lesions and high hematocrit levels (Di Marco et al., 2017; Pichavant et al., 2001). Water salinity in the RAS and IRAS systems ranged between 36 and 37 ppt, which was similar for both treatments and within the tolerance range identified by Laiz-Carrión et al. (2005) for sea bream. The ideal salinity for *Ulva* sp. depends on the species. In Cardoso et al. (2023), it was found that two species of *Ulva* sp. showed better growth rates at different salinity levels, with *U. lacunculata* thriving at 10 ppt and *U. linza* at 30 ppt. In the Ria Formosa lagoon, different types of *Ulva* species have already been identified, such as *Ulva flexuosa*, *Ulva torta*, *Ulva intestinalis*, *Ulva prolifera*, and *Ulva clathrate* (Favot, 2019). This study was conducted using one *Ulva* species, but the lack of information about the specific species used in this trial limits the ability to replicate the experiment and compare the results with other studies. Additionally, not knowing the exact species can lead to variability in environmental tolerance, as different *Ulva* species

may have varying tolerances to conditions such as temperature, salinity, and light (Wang et al., 2018). Furthermore, differences in growth rates and morphology among species can significantly affect the study's outcomes, such as biomass production and nutrient uptake efficiency (Luo et al., 2012).

The performance of *Ulva* sp. in this trial was evaluated for different densities over the weeks. The growth rate and yield of *Ulva* sp. are contingent upon factors including nutrient flow and composition, light conditions (e.g., seasonal variations), climate, and cultivation practices, such as standing stock and flow regime (Neori et al., 2004). In the system, *Ulva* sp. tanks were shaken with strong aeration. Regarding the SGR, it was observed that the density of 1 kg m<sup>-2</sup> obtained the highest results compared to the other densities. These findings confirm the ones by Shpigel et al. (2019), where it was found that the SGR for densities of 1 kg m<sup>-2</sup>, 2 kg m<sup>-2</sup> and 3 kg m<sup>-2</sup> increased as the initial densities decreased, which could be attributed to light limitation influencing the SGR of *Ulva* sp.

The low values of both SGR and Yield observed in the first week may be associated with an adaptation phase of the algae. In this work, it was expected to observe a greater nutrient absorption by the algae, leading to a reduction in the system's nutrients. However, since no significant differences were observed in the nutrients between the two systems, the results reveal that *Ulva* sp. had no direct impact on the nutrients. Nevertheless, despite no differences being observed in the nutrients between the two systems, the hypothesis that this type of IRAS may be optimized or improved cannot be discarded. The amount of *Ulva* sp. used should have been higher relative to the amount of fish that was used, to maximize the assimilation of nutrients by this species of macroalgae. A higher amount of NH<sub>4</sub> was recorded in the fish tanks compared to the other collection sites in both treatments. These results could be explained by the excretion of ammonia by the fish themselves. Fish excrete ammonia through their gills and feces, with the gills being the primary site of ammonia excretion in fish (Weihrauch et al., 2009). In ammoniotelic fishes, most of the ammonia is excreted across the epithelium as NH<sub>3</sub>, down a favorable blood-to-water diffusion gradient, and it quickly transforms to NH<sub>4</sub> when it comes in contact with the water (Evans et al., 2005). When analyzing the nutrients throughout the trial, it was found that the values of NH<sub>4</sub> and NO<sub>3</sub> were significantly higher in weeks six and seven. This may have occurred due to a temperature rise, combined with the increased amount of feed over days, along with the accumulation of organic matter trapped in the central region of the mechanical filters observed in the final phase of the

trial. A rise of approximately 1°C in the last weeks may be associated with an increase in the metabolic rate of the fish and an acceleration in the decomposition of organic matter, resulting in a higher production of ammonia. In the study by Bucking, 2017, it was found that temperature increases lead to a rise in the amount of ammonia excreted by fish. Kinyage & Pedersen (2016) found that higher temperatures, up to 30 °C, increased microbial activity. Results indicated higher ammonia levels in weeks six and seven, allowing the bacterial community time to adapt to these fluctuations. This may be supported by the observed values in week eight, which returned to lower levels, approximately consistent with those in other weeks of the trial. According to Ale et al. (2011), the macroalgae species *Ulva lactuca* demonstrated that the high ammonia values discriminate nitrate absorption by the macroalgae. The assimilation of ammonium and nitrate by seaweed is species-specific (Vonk et al., 2008). In this study, the notable variation in nutrients cannot confirm that the alterations in NO<sub>3</sub> values in the last weeks were associated with the discrimination of the absorption. These variations were equal between treatments, but it was verified that this alteration in the concentration of nutrients significantly reduced the SGR and Yield in week six.

Results regarding the N content showed no differences between the densities, suggesting that N was not a limiting factor for the growth of *Ulva* sp. The availability of this nutrient in the water was in enough concentrations for all the culture conditions. Studies by Neori et al. (1991) and Shpigel et al.(2019) found that N content significantly increased with a higher stocking density, higher N content means they remove more N per unit of biomass, but in this study, there was no significant effect of *Ulva* stocking density on N content. This could be justified due to the physical and spatial limitations, although aerators were used to stir the macroalgae, the accumulation by the end of the sampling days was visible.

*Ulva* sp. was able to efficiently assimilate nutrients, resulting in higher N content in the final phase of the trial compared to the initial phase. The higher N content in *Ulva* sp. at the end of the trial can be related to an increase in the nutrients verified in the last weeks of the trial.

Results during the daylight cycle revealed a higher presence of PO<sub>4</sub> and NO<sub>2</sub> in the IRAS system, and higher NH<sub>4</sub> values during the period of 16h00 to 22h00 for both treatments. These findings contradict expectations and those reported in other studies. Kang (2011) found lower values for all nutrients in systems cultivated with macroalgae

and noted an increase in the values of  $\text{NH}_4$  throughout the day. This increase in  $\text{NH}_4$  could be associated with the excretion patterns of the fish. In the study by Godoy-Olmos (2022), increased levels of ammonia were observed four hours after the first feeding in seabream. In this trial, the fish were fed for the first time at 9h30, followed by feedings at 12h30, 14h30, and 16h30, which could explain why the results showed an increasing pattern after 16h00, with the highest levels observed at 20h00.

The higher  $\text{PO}_4$  and  $\text{NO}_2$  levels in fish tanks belonging to the IRAS system may be associated with the influence of the macroalgae on fish behavior and metabolism. Macroalgae have a direct impact on fish. Most studies show that when introduced in fish feed, they improve fish health and growth (Naiel et al., 2021; Wan et al., 2019). However, this study sought to explore the indirect effects of *Ulva* sp. interaction on fish, as the system was designed to completely exclude the passage of *Ulva* sp. into the fish tanks. Indirectly, the capacity of macroalgae *Ulva* sp. to release secondary metabolites or bioactive compounds into the water could be an important tool in water quality. In Abd El-Baky et al. (2008), the presence of carotenoids and phenolic compounds was confirmed in *Ulva lactuca* when cultivated in saltwater. This species of macroalgae was also used in the study by Pappou et al. (2022) as a source of bioactive compounds, such as carotenoids and phenolic compounds, demonstrating the importance of this species in the release of these compounds. Additionally, these bioactive compounds have a significant impact on fish health. Compounds such as polyphenols and carotenoids are effective free radical scavengers reported to have immunostimulant effects in fish, inducing antioxidant properties and reducing oxidative stress (Citarasu, 2010; Sakai, 1999; Song et al., 2014). In this trial, no effect of these bioactive compounds on fish growth was observed, the influence of the macroalga *Ulva* sp. did not have a direct impact on fish growth, as weight and length were similar between both treatments. Hematological parameters were also similar between RAS and IRAS, between the range of ideal HTC and Hgb values reported for this species and within the values described for seabream for a comparable temperature (Fazio, 2018) and weight (Fazio, 2012). In addition to the antioxidant capacity of bioactive compounds, they can reduce oxidative stress in fish and stimulate hemoglobin production in fish (Naiel et al., 2021). Higher hemoglobin values were expected in the IRAS treatment. It was also expected, as mentioned earlier, an increase in fish performance between treatments given the bio-remediating properties of the macroalgae *Ulva* sp. The enhanced properties of *Ulva* sp. in a system were expected to result in improved fish condition, a healthier and more stable

environment, better growth conditions, and good water quality. These improvements can promote notable differences at hematological and histological levels.

In both systems, the condition of the gills was found to be the same according to a scoring scale classified as mild gill pathology of minor clinical significance, indicating that the presence of macroalgae in the IRAS system did not significantly influence the outcome of fish gill condition. Francis-Floyd (2009) verified that when unionized ammonia reaches a concentration of  $0.05 \text{ mg L}^{-1}$ , gill damage occurred. Gill damage, such as hyperplasia, may be associated with hypoxia situations (Bagherzadeh Lakani et al., 2013). As oxygen levels in both systems were adequate, and results showed ammonia values in the system above  $0.05 \text{ mg L}^{-1}$  in all weeks of treatments, it is assumed that such damages in the gills are associated with high level of nitrogenous compounds. The presence of an aneurysm in only one fish in the RAS system may be associated with possible physical damage during species handling in the samplings and may not manifest as a specific symptom of the RAS treatment.

The lack of significant differences in the performance of the fish between the two systems underscores the importance of conducting a SWOT analysis to identify areas for improvement and optimize the IRAS. A SWOT analysis serves as a critical tool in strategic planning, allowing for the identification and evaluation of Strengths, Weaknesses, Opportunities, and Threats linked to specific projects or business ventures (Garate, 2017).

Starting with strengths, a standout observation from the trial was the rapid growth of macroalgae. This factor can bolster the bioremediation capacity of *Ulva* sp., independent of the lack of significant differences between the two systems. *Ulva* sp. achieved growth in short periods, showcasing its efficiency in nutrient assimilation within the IRAS setup. Interestingly, while the nutrient levels in the water did not vary significantly between treatments, the presence of *Ulva* sp. did not hinder fish growth, underlining its compatibility in aquaculture systems.

As weaknesses, integrating *Ulva* sp. cultivation into the RAS framework brings its own set of challenges. The complexity of operation and management is notably increased, necessitating further expertise, resources, and continuous maintenance to ensure the system's efficacy. Moreover, the marginal improvement in water quality suggests that the lack of optimized fish:seaweed biomass ratios may have hindered the visibility of bioremediation results under our test conditions.

Looking at opportunities, the inclusion of *Ulva* sp. in RAS systems opens promising avenues for innovation and market expansion. This integration could potentially diversify income sources and boost the operation's sustainability, presenting a forward-thinking approach to aquaculture.

However, this integration is not without its threats. The additional costs associated with integrating macroalgae into an automated RAS system, spanning infrastructure, equipment, and personnel, might undermine the project's economic viability and return on investment. Furthermore, this added complexity introduces a higher risk of technical issues, which could disrupt operations and negatively impact the production and quality of aquatic products.

## **5. Conclusions**

To summarize, this study shows that cultivation densities of 1.0 kg FW m<sup>-2</sup> maximized the SGR productivity of *Ulva* sp., with no significance differences in biomass and N yield compared to densities of 1.5 and 2.0 kg FW m<sup>-2</sup>. The results on growth in both fish and macroalgae, and the absence of significant differences between RAS and IRAS in terms of water quality and fish performance, reveal that integrating this species of macroalgae into an already productive system (RAS) is challenging but feasible. The success of using macroalgae in a system depends heavily on the proportion of macroalgae and the fish. Proper fish: seaweed biomass ratios are crucial to ensure that all products in the system are utilized efficiently, creating an IMTA system without any loss of resources. This integration can enhance profitability and promote diverse market opportunities. The potential for improved water quality in IRAS underscores the need for further studies on this species and type of system.

Incorporation of *Ulva* sp. into aquaculture systems not only improves nutrient management but also opens avenues for more sustainable and economically viable aquaculture practices. Future research should focus on optimizing fish: seaweed biomass ratios and exploring long-term impacts on both water quality and overall system productivity. This approach underscores the potential of IRAS systems to revolutionize aquaculture, promoting sustainability and resource efficiency in the industry.

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