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Development and optimization of seaweed cultivation in IMTA

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Development and optimization of seaweed cultivation in IMTA

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Declaração de autoria de trabalho

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

Jéni Cândido Soares

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Abstract

In aquaculture, discharges of wastewater from fed species can lead to eutrophication of the receiving aquatic environment. The nutrient-rich wastewater can be reused in Integrated Multi-Trophic Aquaculture (IMTA), which enhances cultivation efficiency by combining species from different trophic levels to minimize waste. The macroalgae have numerous benefits, and their cultivation in aquaculture wastewater results in more sustainable and profitable aquaculture.

This study involved two experiments. The first evaluated the effect of cultivation density and method (with and without substrate – 2 mm twisted nylon twine) on growth of *Codium tomentosum* and *Halopteris scoparia* under controlled conditions (18°C, light intensity of 70-100 μmol photons m^{-2} s^{-1}, with a 12:12 photoperiod) over six weeks. The second evaluated the cultivation and bioremediation potential of *H. scoparia* in an IMTA system, analyzing the effect of four cultivation densities (5, 10, 15, and 20 g/L) over nine weeks.

In the first experiment, both cultivation methods and species experienced significant contamination by epiphytic algae. In tumble cultivation, *C. tomentosum* had a higher SGR at 4 g/L, while 8 g/L had a higher average yield. *H. scoparia* showed higher SGR and yield at 2 g/L but greater average length at 4 g/L. Regarding substrate cultivation, according to visual observation, there may have been attachment of *C. tomentosum*; however, longer cultivation is required to confirm it. In contrast, *H. scoparia* did not demonstrate attachment or development of new individuals from spores.

In *H. scoparia* cultivation within an IMTA system, the lowest densities (5 and 10 g/L) had the highest average SGR and yield but also a higher presence of epiphytic algae, potentially explaining the increased values. These densities also showed significantly higher N-yield. In contrast, the higher densities (15 and 20 g/L) had a significantly higher C:N ratio, suggesting nitrogen limitations that may have affected growth.

Keywords: Bioremediation, *Codium tomentosum*, *Halopteris scoparia*, Integrated Multitrophic Aquaculture (IMTA), macroalgae cultivation.

Resumo

Na aquacultura, a descarga das águas residuais provenientes do cultivo de espécies alimentadas, como os peixes, pode causar a eutrofização do meio aquático recetor. Além disso, pode representar um desperdício de recursos, uma vez que essa água é rica em nutrientes e pode ser utilizada para o cultivo de outras espécies, como as macroalgas. A Aquacultura Multitrófica Integrada (IMTA) associa o cultivo de duas ou mais espécies aquáticas de diferentes níveis tróficos, de modo a aumentar a eficiência do cultivo e minimizar os resíduos. Nos últimos anos, os benefícios das macroalgas, têm sido bem conhecidos e documentados, como por exemplo, o seu uso como fertilizantes, o seu valor nutricional, as suas propriedades farmacêuticas e a sua utilização na indústria cosmética. O cultivo de macroalgas em águas residuais da aquacultura de peixes pode resultar numa aquacultura mais sustentável e eficiente, além de proteger o meio ambiente e gerar mais lucros a partir da nova biomassa.

Neste trabalho foram realizadas duas experiências. A primeira teve como objetivo avaliar o potencial de cultivo de *Halopteris scoparia* e *Codium tomentosum* em condições ambientais controladas (18°C, intensidade luminosa de 70-100 μmol fotões m^{-2} s^{-1}, com um fotoperíodo de 12:12). As macroalgas foram cultivadas sem substrato (cultivo com agitação/tumble) e com substrato (cordão de nylon torcido de 2 mm), em duas densidades de cultivo para cada espécie e método de cultivo, durante 6 semanas. Nesta experiência investigou-se como as densidades e o método de cultivo afetam o crescimento destas espécies. No cultivo de *H. scoparia*, foram usadas as densidades de 2 g/L e 4 g/L para ambos os métodos de cultivo. No cultivo de *C. tomentosum*, as densidades foram de 4 g/L e 8 g/L para o cultivo sem substrato, e de 33 g/L e 65 g/L para o cultivo em substrato. Por fim, a segunda experiência teve como objetivo avaliar o potencial de cultivo e biorremediação de *H. scoparia* em um sistema IMTA ao longo de 9 semanas, analisando o efeito de quatro densidades de cultivo (5, 10, 15 e 20 g/L).

Relativamente à primeira experiência, tanto o cultivo de *C. tomentosum* quanto de *H. scoparia* mostraram variações significativas no crescimento, dependendo do método e densidade de cultivo. No cultivo sem substrato de *C. tomentosum*, obteve-se maiores valores médios de SGR (% FW d^{-1}) na densidade de 4 g/L (7.35 ± 3.96) em comparação com a de 8 g/L (4.60 ± 1.80), com diferenças significativas entre densidades ao longo do cultivo. Contrariamente, a densidade de 8g/L (3.06 ± 1.31) obteve maiores valores médios de yield (*g* FW L wk^{-1}) do que a de 4 g/L (2.82 ± 1.75), mas sem diferenças significativas. Ambas as densidades apresentaram uma elevada contaminação por outras algas, mas visualmente a densidade de 4 g/L aparentou ter maior incidência de contaminação. Por outro lado, o comprimento médio (cm) da macroalga ao longo da experiência, foi semelhante entre as densidades, sendo 1.29 ± 0.25 (4 g/L) e 1.27 ± 0.25 (8 g/L), sem diferenças significativas entre densidades ao longo do cultivo. No cultivo sem substrato de *H. scoparia*, os valores médios de SGR (% FW d^{-1}) e yield (*g* FW L wk^{-1}), foram superiores na densidade de 2 g/L (10.18 ± 4.32 e 2.08 ± 1.08 respetivamente) comparativamente à densidade de 4 g/L (5.59 ± 2.59 e 1.93 ± 1.04, respetivamente). No entanto, também foi a densidade de 2 g/L que apresentou, visualmente, mais incidência de contaminação. Por outro lado, a densidade de 4 g/L (0.82 ± 0.37) obteve valores médios de comprimento (cm) superiores à de 2 g/L (0.75 ± 0.37), com diferenças significativas ao longo do cultivo.

No que diz respeito ao cultivo em substrato de *C. tomentosum*, a observação visual indica que a densidade mais alta apresenta mais pontos verdes (que se presume serem *C. tomentosum)*, sendo a mais eficaz. No entanto, é necessário um cultivo mais longo de modo a

se confirmar que é *C. tomentosum*. Em contrapartida, no cultivo de *H. scoparia*, a macroalga esteve durante duas semanas no meio para libertar os esporos, no entanto não se identificou a sua fixação ao substrato. Para ambas as espécies, houve contaminação dos cordéis por outras algas em ambas as densidades; no entanto, as densidades mais baixas aparentaram, visualmente, ter maior incidência de contaminação.

Por fim, no cultivo de *H. scoparia* em sistema de IMTA, as densidades mais baixas foram as que apresentaram maiores valores médios de SGR (% FW d^{-1}) e yield ($g\ FW\ m^2\ wk^{-1}$). Sendo esses, respetivamente, 8.42 ± 2.07 e 819.45 ± 252.33 (5 g/L); 5.11 ± 1.05 e 880.77 ± 208.53 (10 g/L); e os das densidades mais altas 2.72 ± 1.25 e 602.26 ± 269.28 (15 g/L) e 1.19 ± 1.45 e 351.66 ± 431.40 (20 g/L). Além disso, 5 g/L e 10 g/L apresentaram os valores mais elevados de conteúdo de azoto (%N) na macroalga e N-yield. No entanto, também foram estas densidades que revelaram uma maior contaminação por epífitas, o que pode ter explicado os valores mais elevados de SGR e yield. Adicionalmente, as densidades de 15 g/L e 20 g/L apresentaram uma relação C:N significantemente mais elevada do que as densidades menores, sugerindo potenciais limitações de azoto o que pode ter afetado o crescimento de *H. scoparia* nestas densidades. Com base em todos os resultados, e perante estas condições de cultivo, a densidade de 10 g/L foi a que pareceu ser a melhor densidade de cultivo, uma vez que apresentou vários valores mais elevados de SGR, yield e um N-yield significativamente mais elevado. No entanto são necessários mais estudos. Por fim, todas as densidades obtiveram valores de teor de %N nos tecidos superiores a vários estudos mencionados, o que demonstra que *H. scoparia* poderá vir a ser uma boa opção de biorremediação de azoto e cultivo em IMTA.

Estes resultados demonstram que o principal desafio em todos os métodos de cultivo foi a contaminação por algas epífitas, evidenciando a grande necessidade de se colher macroalgas sem contaminação e/ou desenvolver métodos de limpeza mais eficientes. Além disso, os resultados revelaram que são necessários mais estudos para se otimizar o cultivo de ambas as espécies.

Palavras-chave: Biorremediação, *Codium tomentosum*, *Halopteris scoparia*, Aquacultura Multitrófica Integrada (IMTA), cultivo de macroalgas.

Index

Index of figures

Index of tables

Abbreviations

%	Percentage
µmol	Micromole
°C	Degrees Celsius
µM	Micromolar
ANOVA	Analysis of variance
C	Carbon
cm	Centimetre
CO_2	Carbon dioxide
d	Day
DW	Dry Weight
EPPO-IPMA	Estação Piloto de Piscicultura de Olhão – Instituto Português do Mar e Atmosfera
FW	Fresh Weight
g	Gram
GeO_2	Germanium dioxide
h	Hour
IMTA	Integrated Multitrophic Aquaculture
L	Litre
L:D	Light:Dark
m	Meter
min	Minutes
mL	Milliliters
mm	Millimetre
N	Nitrogen
n	Number
NH_3	Ammonia
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
PO_4^{3-}	Phosphate
PSU	Practical Salinity Units
RGR	Relative Growth Rate
s	Second
SD	Standard deviation
SGR	Specific Growth Rate
UV	Ultraviolet
Wk	Week
Y	Yield

1. Introduction

1.1. Aquaculture and Integrated Multi-Trophic Aquaculture

Aquaculture, as defined by the Food and Agriculture Organization of the United Nations (FAO), consists of the farming of aquatic organisms, including fish, mollusks, crustaceans, and aquatic plants. This practice emerged as a response to overfishing and the growing demand for aquatic products. Nevertheless, the intensive development of aquaculture has raised significant environmental concerns, like the excessive use of resources (such as water, feed, fertilizers, chemicals, and energy), the dependence on commercial feed, and the discharge of its effluents (Granada *et al.*, 2015; Shpigel, 2012).

The feed used in aquaculture is usually high in protein and therefore contains high amounts of nitrogen and phosphorus (Boyd *et al*., 2022; Munguti *et al*., 2020). However, the retention of these nutrients ranges between 10-49% (nitrogen) and 20-40% (phosphorous); the rest is lost by the fish metabolism (feces and gills) and uneaten food, which then dissolves and releases the nutrients (Granada *et al.*, 2015; Grigorakis & Rigos, 2011; Yogev *et al*., 2020; Shpigel, 2015). These retention percentages depend on the species of fish, its growth rate, the type of feed provided, and its ingredients (Dauda *et al.*, 2019; Granada *et al.*, 2015; Munguti *et al*., 2020; Shpigel, 2015).

Aquaculture effluents contain a variety of components, including solid waste, such as feces and uneaten food, as well as dissolved waste (e.g., CO_2, NH_3, and PO_4^{3-}) from fish metabolism or the decomposition of uneaten fresh food (Cao *et al.*, 2007; Dauda *et al.*, 2019). The main end product of protein metabolism in fish is ammonia (NH_3), which can cause an increase in biochemical oxygen demand and is toxic to the fish, so it must be removed quickly or converted into a less toxic substance, nitrate (NO_3^-) (Boyd, 2018; Cao *et al.*, 2007; Porter *et al.*, 1987). The quality and quantity of the wastewater from aquaculture depend on the cultivated species, stocking density, farming system, feed quality (feed ingredients), the amount of uneaten feed, which depends on feeding management, and whether feeding is automatic or manual (Grigorakis & Rigos, 2011; Pillay, 2004; Wu, 1995).

The release of nutrient-rich aquaculture effluent into the environment can lead to oxygen depletion, eutrophication, algae blooms, changes in biomass and community structure, the appearance of diseases, and habitat destruction (Cao *et al.*, 2007; Yang *et al.*, 2020). Additionally, discharging such wastewater can not only destroy ecosystems but also represents a waste of resources and financial loss, as this nutrient-laden water could be repurposed for cultivating other organisms, turning a potential waste into profitable and sustainable practices (Shpigel *et al.*, 2018).

The aquaculture effluent can be used to grow macroalgae. Macroalgae can act as a biofilter, consuming nitrogen (NH_4^+, NO_3^-, NO_2^-), phosphate (PO_4^{3-}), and other nutrients present in aquaculture effluent and transforming it into the value-added biomass that is macroalgae (Chopin & Wagey, 1999; Krom & Neori, 1989; Neori *et al.*, 2004; Yang *et al.*, 2020). Through photosynthesis, macroalgae absorb carbon dioxide and produce dissolved oxygen, thus balancing fish oxygen demand and oxygen depletion problems (Krom & Neori, 1989; Yang *et al.*, 2020). The algae-cleansed effluent could be recirculated into the fish tanks or discharged into the environment without affecting the surrounding ecosystems (Neori *et al.*, 2004). The cultivation of macroalgae with the effluent of fed organisms is known as Integrated Multi-Trophic Aquaculture (IMTA) (Chopin, 2012).

Integrated Multi-Trophic Aquaculture is an approach that involves cultivating species from different trophic levels with complementary ecosystem functions to benefit from synergistic interactions between species (Chopin, 2012; Troell *et al.*, 2003). Within IMTA, some organisms, such as fish and shrimp, are fed artificially, while others, such as bivalve mollusks or seaweed, extract their food and nutrients from the environment (Chopin, 2012; Neori *et al.*, 2004). This process facilitates biomitigation by partially removing nutrients resulting in commercially valuable, harvestable, and healthful seafood (Chopin, 2012; Troell *et al.*, 2003).

The IMTA has emerged as a sustainable practice in aquaculture, offering environmental benefits and increased ecological efficiency and profitability. By reducing the discharge of nutrient-rich aquaculture effluents into the environment and enabling the cultivation of diverse species without the need for additional resources, IMTA contributes to a more environmentally friendly and economically stable industry. This approach not only enhances ecosystem health through biomitigative services but also fosters job creation, lowers production costs, and promotes product diversification. Moreover, IMTA facilitates better regulatory governance, improved management practices, and the creation of differentiated and safe products, garnering social acceptance in the process (Chopin, 2012; Neori *et al.*, 2004; Troell *et al.*, 2003). Additionally, IMTA can be applied in closed, semi-closed, or flow-through systems in all types of waters (cold, warm, and temperate) and in all culture systems (extensive, semi-intensive, and intensive) in sea cages, land-based facilities, and freshwater in land-based facilities or ponds (Shpigel, 2015).

1.2. Macroalgae

The multicellular algae are called macroalgae, as well as seaweed, which lacks a formal definition but frequently refers to marine algae (Neori *et al.*, 2004; Pereira, 2015; Sahu, *et al.*, 2020). Macroalgae belong to the domain Eukarya and are classified within the kingdoms Plantae and Chromista (Pereira, 2015). The kingdoms can be classified into phyla and then into classes based on their pigmentation. The kingdom Plantae divides into two phyla, the Chlorophyta (green algae) with marine macroalgae in the Ulvophyceae class and the Rhodophyta (red algae) with mostly macroalgae in the Bangiophyceae and Florideophyceae classes. The kingdom Chromista devides in the phyla Ochrophyta (brown algae) with macroalgae in the Phaeophyceae class (Pereira, 2015). Green macroalgae have as photosynthetic pigments chlorophylls a and b and carotenoids (identical to the plants); the red macroalgae pigments are chlorophyll a, phycobilins (phycocyanin and phycoerythrin) and carotenoids (β-carotene, lutein, and zeaxanthin); finally, the brown macroalgae have as photosynthetic pigments chlorophylls a and c and carotenoids (predominantly fucoxanthin) (Pereira, 2009).

Macroalgae can grow to over 50 meters and exhibit different morphologies with distinct thallus and ramifications (Pereira, 2009). Have a wide geographic distribution, are present in diverse habitats, and can be found floating, attached, or associated with other organisms (epiphytism, endophytism) (Pereira, 2015; Sahoo & Seckbach, 2015). A number of applications have been identified for macroalgae. For instance, can be consumed as a direct food in fresh, dried, pickled, or cooked forms (Pereira, 2009; Pereira, 2011) and have an increased nutritional interest since they contain essential vitamins and minerals and contain a high proportion of polyunsaturated fatty acids (Ito & Hori, 1989; Pereira *et al.*, 2012; Yang *et al.*, 2020). They can also be used for fertilizers; the extraction of phycocolloids (agar, carrageenans, and alginates); the extraction of compounds with antiviral, antibacterial, or antitumor action; and as bioindicators for assessing the ecological status of water bodies (Pereira, 2009).

Macroalgae are primary producers (photoautotrophs organisms), meaning that algae have cells with pigments that allow them to perform photosynthesis, using sunlight as energy, converting inorganic nutrients (such as carbon, nitrogen, and phosphorus) present in the water, and producing oxygen and new biomass (Neori *et al.*, 2004; Pereira, 2015; Sahoo & Seckbach, 2015). For example, for IMTA system algae that can tolerate high levels of ammonium (NH_4^+) are advantageous (Pereira *et al.*, 2008). Several aspects must be considered when cultivating macroalgae, such as high growth rate, ease of culture, life cycle control, nitrogen concentration in the tissue, resistance to epiphytes, and disease-causing organisms (Neori *et al.*, 2004).

1.3. Macroalgae cultivation

Understanding the interplay of abiotic and biological factors is crucial for optimal macroalgae growth. Temperature and light influence photosynthesis and other metabolic activities in macroalgae, affecting their growth and productivity (Balar & Mantri, 2020; Demetropoulos & Langdon, 2004). The seasonal variations affect growth, with some species achieving a higher growth rate during the summer (significantly correlated to temperature) (Balar & Mantri, 2020). While others achieved higher yields in spring and lower in summer due to stressors like high temperature and light. In this case, in summer the concentrations of pigments that capture light may be lower and the accessory xanthophylls, which are responsible for photoprotection higher, indicating seasonal photoacclimatization (Marques *et al.*, 2022). The photoperiod also has a significantly impact on growth, with longer days enhancing growth rates (Marques *et al.*, 2022). Nevertheless, the geographic distribution can influence growth rates and temperature tolerance within the same species (Novaczek *et al.*, 1989). Macroalgae can grow across various salinities, but their survival and optimal growth are often favored at specific salinities, which can be temperature-dependent (Hanisak, 1979a; Silva, 2011). Stocking density is another important factor; higher densities lead to increased nutrient competition and self-shading, reducing photosynthesis and growth rates; however, productivity can be higher at these densities (Abreu *et al.*, 2011; Demetropoulos & Langdon, 2004; Kim *et al.*, 2013; Pereira *et al.*, 2006). Additionally, photosynthesis and growth can be affected or completely inhibited at high pH levels (>8.5, >9, or >9.5) depending on the species (Blinks, 1963; Lise & Juel, 2007; Zou & Gao, 2009).

The main elements required by macroalgae are nitrogen, inorganic carbon, and phosphorus, which can limit their photosynthesis and growth (Roleda & Hurd, 2019; Sahoo & Seckbach, 2015; Sheppard *et al.*, 2023). Nutrient limitation in seaweeds occurs when the demand for a nutrient exceeds its availability, thereby restricting growth (Harrison & Hurd, 2001; Roleda & Hurd, 2019). The biochemical composition (C, N, and P) and the atomic C:N or N:P ratios in macroalgae biomass are commonly used to assess the nutritional status; when the C:N values are higher, it suggests nitrogen limitation (Gómez Pinchetti *et al.*, 1998; Hanisak, 1990; Vergara *et al.*, 1993). Additionally, some species may experience carbon limitation because they cannot use bicarbonate (HCO_3^-) as an inorganic carbon source (Roleda & Hurd, 2019). Furthermore, the capacity to use bicarbonate (HCO_3^-) can be reduced during conditions of very high pH (often above 9.0), which can affect photosynthetic rates and biomass production (Zou & Gao, 2009; Zou *et al.*, 2004). Phosphorus is an essential nutrient in fish feeds present in aquaculture´s effluents that can impact ecosystems (Global Seafood Alliance, 2023). The presence of phosphorus in the water increases the amount of phosphate (PO_4^{3-}), which is the best form of phosphorus for seaweed growth (Troell *et al.*, 2003). The nutrient uptake rates in macroalgae depend on nutrient availability and environmental conditions, with variation in saturation velocity based on species requirements, morphology, and growth form (Roleda & Hurd, 2019).

Various studies report that also depending on the species, the macroalgae may or not absorb different sources of nitrogen and may preferentially remove one nitrogen source (Ahn *et al.*, 1998; Fries, 1963; Kang *et al.*, 2011; Neori *et al.*, 1996; Prince, 1974). Regarding the abiotic factors, according to Harrison & Hurd (2001), ammonium uptake may be less dependent on light than nitrate uptake. Kang et al. (2008) found that ammonium removal efficiency was significantly higher under specific irradiance and temperature, with this temperature varying according to the initial ammonium concentrations. Moreover, abiotic factors, such as temperature, light, photoperiod, and salinity, can affect the life stage of the macroalgae (Balar & Mantri, 2020). Consecutively, these life stages can affect their growth and nutrient uptake. For example, Thomas et al. (1985) reported that the ability of $N\text{-}NH_4^+$ to inhibit $N\text{-}NO_3^-$ appeared to differ depending on the life stage, and Ohtake et al. (2020) reported that P dynamics vary depending on the life stage.

Lastly, in macroalgae cultivation, contamination by epiphytic algae is a frequent problem. Epiphytic algae use the tank´s surface and/or cultivated macroalgae as substrates and compete for nutrients and light (Neori *et al.*, 2004), which can lead to lower growth of the cultivated macroalgae and lower quality biomass production (Sahu *et al.*, 2020). Normally, higher irradiances and amounts of nutrients were coincident with higher amounts of epiphytes (Kersen *et al.*, 2011; Marques *et al.*, 2022; Patarra *et al.*, 2017). When contamination by epiphytes becomes significant, it may be necessary to renew all the biomass and, subsequently, restock new clean biomass in clean tanks (Neori *et al.*, 2004), causing losses in biomass and profit (Ganesan *et al.*, 2015; Vairappan *et al.*, 2008). Therefore, effective management of macroalgae cultivation to reduce epiphytes is crucial. Thus, various methods can be carried out to prevent and/or combat epiphytic algae, including biological, physical, and chemical approaches, to ensure the productivity of macroalgae cultivation (Sahu *et al.*, 2020). Physically, epiphytes can be removed manually through mechanical brushing, rapid water movement, or even manually using forceps (Sahu *et al.*, 2020; Vairappan *et al.*, 2008). Chemical procedures could be done by changing the pH or by rinsing using chlorine or copper (Sahu *et al.*, 2020). In this case, fleshy macroalgae are more resistant to chemicals than thin macroalgae, which can be advantageous for solving problems with fine epiphytes (Neori *et al.*, 2004). Another preventive chemical procedure is to place the macroalgae in a solution (autoclaved seawater) with 10% betadine (Carreiro, 2014). Additionally, reducing epiphytic algae outbreaks can be achieved by adopting certain practices, such as filtering and UV-treating the incoming water and controlling environmental conditions (e.g., reducing irradiance levels, the amount of nutrients present in the water, temperature, and salinity) (Fong *et al.*, 1997; Ganesan *et al.*, 2015; Kersen *et al.*, 2011; Patarra *et al.*, 2017; Sahu *et al.*, 2020; Vairappan, 2006). Other strategies include increasing cultivation densities and making slight and frequent changes in the water level (e.g., partially drying out the biomass, affecting only the epiphytes) (Lüning & Pang, 2003; Neori *et al.*, 2004). Finally, it is important to monitor the progress of cultivation to identify early signs of epiphyte outbreaks (Vairappan *et al.*, 2008).

1.4. Macroalgae species used in this study

1.4.1. *Codium tomentosum* Stackhouse 17978

Codium is a genus of green algae and includes 139 accepted species names (Guiry & Guiry, 2011). C*odium* grows on rocky and sandy habitats and is widely distributed, so it may be found in both temperate and tropical marine waters (Goff *et al.*, 1992; González & Santelices, 2004). *Codium tomentosum* is native to the northeast Atlantic Ocean from the British Isles southward to the Azores and Cape Verde (Pereira, 2015) and is also known as Chorão, Chorão-do-mar, Pingarelhos (Freitas *et al.*, 2021). It may be found southward to Morocco and Algeria (Silva, 1955). In Portugal, this species is very common along the entire coast, being present in both sheltered and exposed locations, such as tide pools, the lower horizon of the mediolittoral shelf, and the upper horizon of the infralittoral shelf (Pereira, 2009).

Morphologically, *Codium* spp. represented in Figure 1.1, is dark green with a spongy consistency (Freitas *et al.*, 2021; Pereira, 2009). The thallus is covered with colorless hairiness, which can be seen when the algae is immersed in water (Freitas *et al.*, 2021; Pereira, 2015). Grows upright, developing a dichotomously branched cylindrical or subcylindrical thallus, which is attached to the substratum by a small disk, the holdfast, made up of numerous fine threads (González & Santelices, 2004; Pereira, 2015). It is spongy thallus contains many chloroplasts and an internal structure of intertwined colorless medullary filaments (Brawley & Johnson, 1992), has no cellular organization, and is formed by parallel siphons (utricles) surrounding a central bundle of filaments (Freitas *et al.*, 2021).

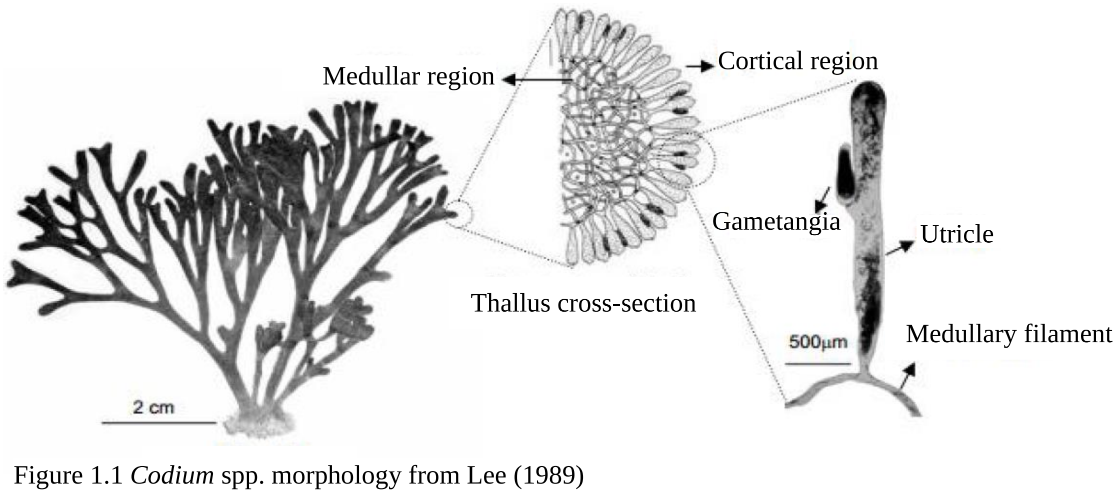

Figure 1.1 *Codium* spp. morphology from Lee (1989)

Most species of the genus *Codium* present the typical characteristics of *C. tomentosum* (Pereira, 2009), so this species can be confused with others. As an example, *Codium fragile,* an exotic species already seen in southern Portugal and quite abundant throughout the European coast, that can be confused with *C. tomentosum* (Mata, 2020; Pereira, 2015). However, the other species can be distinguished by the morphological characteristics of *C. tomentosum* because it has flattening at the level of dichotomies, with 1 to 1.5 cm wide and by it is utricles that are mucronate, that is, no pointed tips (Pereira, 2009; Trowbridge *et al.*, 2004). In the late summer and early fall, when temperatures are warm, *Codium* grows (Kang *et al.*, 2008). In autumn, the thallus of this alga can reach sizes of 30 to 50 cm in length and can be covered by epiphytic algae (Pereira, 2009).

1.4.1.1. Life Cycle

The *Codium* genus has a diplontic life cycle with gametic meiosis and is frequently dioecious (Miravalles *et al.*, 2012), meaning that both male and female individuals exist. Sexual reproduction, represented in Figure 1.2, begins when gametes are formed from the male and female gametangia that are produced in the utricles (Miravalles *et al.*, 2012; Nanba *et al.*, 2005). Both male and female gametes are biflagellate, with the female gametes being larger, about 20 μm long, and the male gametes being smaller, 3 to 4 μm long, and elongated (Borden & Stein, 1969). The zygote is created when the gametes fuse, and it develops into a siphon filament (Miravalles *et al.*, 2012).

Besides sexual reproduction, the capacity for asexual reproduction, through parthenogenesis development, has been described for *C. fragile*, its subspecies (Nanba *et al.*, 2005) and also *C. tomentosum* (Miravalles *et al.*, 2012). For *C. fragile* and its subspecies and *C. fragile subsp. tomentosoides*, vegetative reproduction through thallus fragmentation has also been described (Mata, 2020; Nanba *et al.*, 2005).

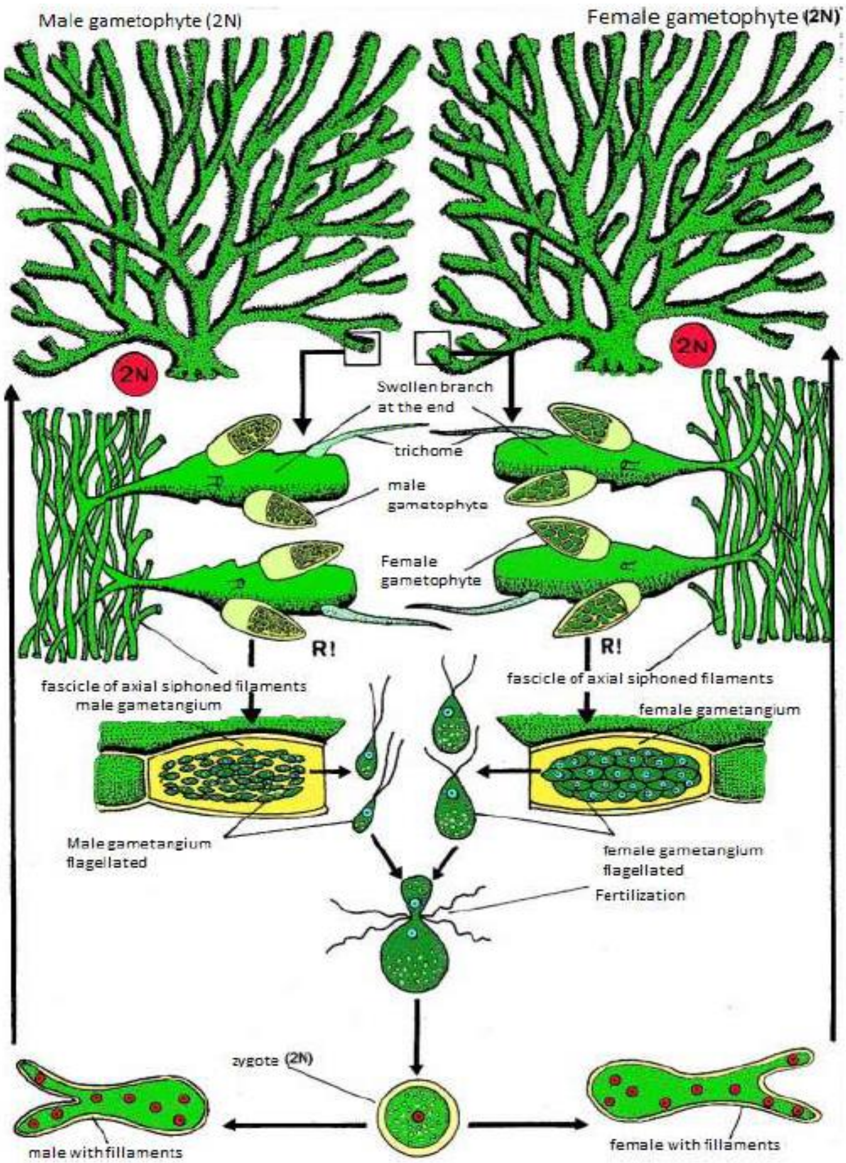

Figure 1.2 Life cycle of *Codium tomentosum*, adapted from Williams as seen in Mata (2020).

1.4.1.2. Applications

In Portugal, Spain, and other countries, *Codium tomentosum* is cultivated because it is considered an edible species (Freitas *et al.*, 2021). *Codium* spp. have been used as human food (Kang *et al.*, 2008) and *C. tomentosum*, is consumed fresh in Malaysia, India and Japan, used in confectionary teas in India, and in Japan is dried and preserved in salt or cooked and add to soups (Freitas *et al.*, 2021). *Codium* spp. have potent antifungal activities (Ballesteros *et al.*, 1992) and *C. tomentosum* has been shown to be an interesting source of neuroprotective and anti-inflammatory agents (Silva *et al.*, 2020; Silva *et al.*, 2021). In the cosmetic industry, extracts of *C. tomentosum* can be used since it regulates the distribution of water in the skin and has the capacity to repair and protect the skin from dryness (Wang *et al.,* 2015). Christabell (2011) showed that *C. tomentosum* had more antibacterial activity against Gram negative bacteria than Gram positive bacteria. According to da Costa (2015), from the perspective of polar lipid content, *C. tomentosum* from IMTA may be viewed as a desirable cash crop since it is a promising element for both animal and human nutrition and exhibits a variety of possible health advantages for humans.

1.4.1.3. Cultivation

Research on *Codium* has been ongoing for several years. However, studies on the species *C. tomentosum* are scarce compared to the species *C. fragile*, which belongs to the same genus (Guiry & Guiry, 2022). As a result, some research on this species was also discussed to provide more information.

According to four investigations on different *Codium* species, the highest growth rate was obtained between temperatures of 17–24 ºC, irradiance of 28–230 µmol photons m^{-2} s^{-1}, salinity of 24–31, and a photoperiod of 16L:8D (Hanisak, 1979a; Hwang *et al.*, 2005; Marques *et al.*, 2021; Yang *et al.*, 1997). Notably, for *Codium tomentosum*, two studies obtained the optimal growth at 20 ºC and 16L:8D (Marques *et al.*, 2021; Yang *et al.*, 1997). Moreover, Hanisak (1979a) obtained that *Codium fragile* spp. *tomentosoides* exhibited its greatest growth during the summer, with a significant correlation to temperature; also, multiple models suggested an interaction between temperature and irradiance. Marques (2022) observed that for *C. tomentosum*, high light exposure in spring increased yields, but in summer, when both temperature and irradiance were higher, yields decreased.

Regarding the nitrogen intake of *C. fragile*, it was found to be highest in the winter and lowest in the summer, suggesting nitrogen storage during winter to support new growth in spring (Hanisak, 1979b). Also, *Codium* can absorb all three forms of nitrogen simultaneously, with uptake being higher at 12–24 ºC. However, the presence of ammonium (NH_4^+) reduces the uptake of the other two forms (Hanisak & Harlin, 1978). Additionally, Kang et al. (2008) found that ammonium removal efficiency was significantly higher under irradiance of 100 µmol photons m^{-2} s^{-1} at 20 and 25 °C, with initial ammonium concentrations of 150 and 300 µM of NH_4^+.

About substrate cultivation, Hwang et al. (2005) obtained the maximum growth of *C. fragile* (Suringar) Hariot cultivated by zygotes attachment (sexual reproduction) under 15 ºC and 20 μmol photons m^{-2} s^{-1} after 15 days of culture. Additionally, Nanba et al. (2005) found that the combination of water flow and irradiance influences the formation and growth of spongy and filamentous thalli, and only the water flow affects the formation of filamentous thalli. Finally, it was found that the depth of the cultivation rope could be able to control the optimal growth of *C. fragile* due to the irradiance changes with the depth (Hwang *et al*., 2008). This last result corroborates the results of Nanba et al. (2005).

1.4.2. *Halopteris scoparia*

Halopteris scoparia (Linnaeus) Sauvageau (1903), previously known as *Stypocaulon scoparium* (Draisma *et al.*, 2010), belongs to the genus *Halopteris* and family Stypocaulaceae (Guiry & Guiry, 2022). *Halopteris scoparia* (Figure 1.3) is a dark brown alga, consisting of very branched (Sánchez Moyano, 1996; Neto *et al.*, 2005), (alternately branching) and very rough filaments (Freitas *et al.*, 2021). It grows into stiff, upright, compact tufts that range from 3 to 15 cm tall and that attach to the substrate through rhizoids that emerge from the axes to form a kind of small to extensive spongy/fibrous discs (Neto, 1997; Neto *et al.*, 2005; Pereira, 2015; Sánchez Moyano, 1996). According to Sánchez Moyano (1996), the morphology and size of the thallus of *H. scoparia* change throughout the year, with a more compact and branched appearance in summer than in winter.

This species is perennial and grows in protected areas (Pereira, 2015). It can be found on the coast, on rocks and sandy bottoms, in intertidal pools, and epiphytically on other seaweeds (Pereira, 2015; Sánchez Moyano, 1996). *H. scoparia* is a widely distributed seaweed. It has been found from the coasts of Norway to Nigeria, the Red Sea, the Caspian Sea, Algeciras Bay, Canada, and the Pacific Northwest (Sánchez Moyano, 1996). In Portugal, *H. scoparia* is found on rocks more or less covered with sand in places with calm waters on the mediolittoral plateau (Pereira, 2009).

Figure 1.3 Morphology of *Halopteris scoparia*. Harvey's original illustration (Phycologia Britannica), as seen in MACOI.

1.4.2.1. Life cycle

Since there is a lack of information about the life cycle of *Halopteris scoparia*, the life cycle of *Halopteris dura*, a species of the same genus, is presented. Kawai and Reine (1998) investigated the life cycle of *Stypocaulon durum* (Accepted name *Halopteris dura*). According to the findings of this study, this species is dioecious and has an isomorphic life cycle, as represented in Figure 1.4. The sporophytes produce unilocular sporangia formed in clusters (Figure 1.5 A), these unispores develop into gametophytes, forming plurilocular organs. The gametophytes produce plurilocular macro-gametangia (female represented in Figure 1.5 B) and plurilocular micro-gametangia (male represented in Figure 1.5 C) and oospores parthenogenetically develop into sporophytes (Kawai & Reine, 1998). According to Kawai and Reine (1998), male gametes were not released neither germinated in situ, therefore, it was considered that these male gametangia might be residual and not functional.

Gibson (2013) studied the reproduction and life-history of the Sphacelariales and concluded that sexual reproduction is rare in most species, while asexual reproduction is common in the order through vegetative means production of asexual spores or by parthenogenesis. Therefore, *Halopteris scoparia* is recognized to have an exceptional capacity to regenerate apical cells, and fragments were shown to grow easily in culture (Gibson, 2013; Higgins, 1931). Furthermore, Neto (1997) reported that *Halopteris scoparia* has unilocular, oval-shaped sporangia.

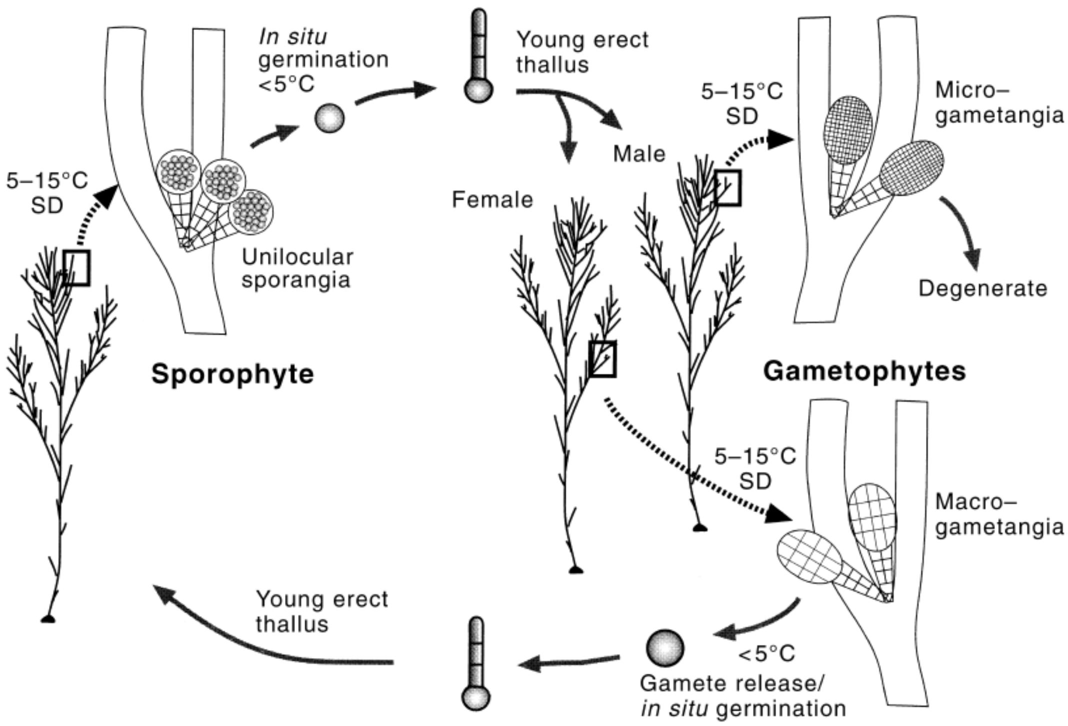

Figure 1.4 Life cycle of *Halopteris dura*, adapted from Kawai and Reine (1998).

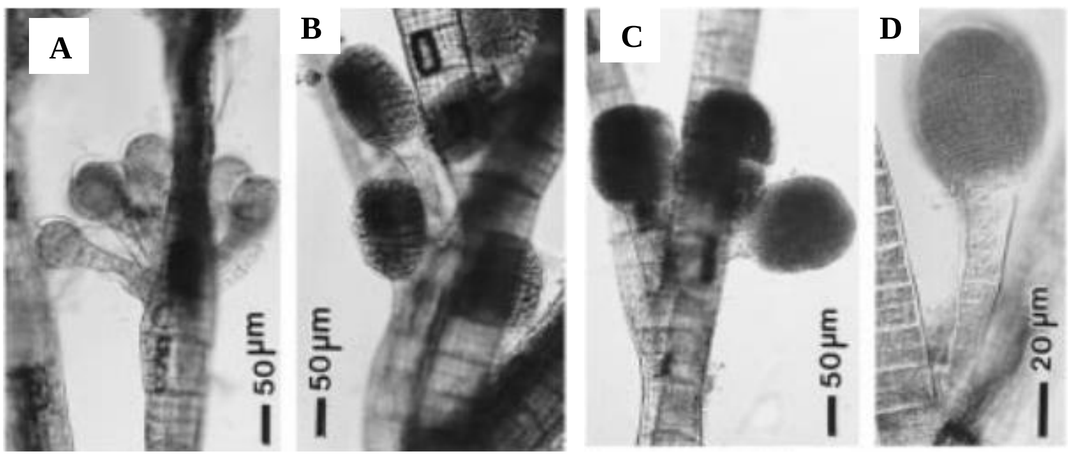

Figure 1.5 Reproductive structures of *Halopteris dura*. A) Unilocular sporangia formed in clusters. B) Macrogametangia (female gametangia) formed in clusters. C) and D) Microgametangia (male gametangia). Adapted from Kawai and Reine (1998).

1.4.2.2. Applications

Several uses have already been demonstrated for the genus *Halopteris* and the species *H. scoparia*. *Halopteris* spp. can be used directly as food for the spider crab *Leucippa pentagona* (Varisco *et al.*, 2015) and has been proven to have antibacterial (Taskin *et al.*, 2007) and antifungal (Ballesteros *et al.*, 1992) properties. *H. scoparia* can also be used directly as food for the sea urchin *Paracentrodus lividus* (Murillo-Navarro & Jiménez-Guirado, 2012), the sea spider *Ammothella longipes* (Soler-Membrives *et al.*, 2011) and has been shown to have antimicrobial (Almeida, 2007), antioxidant (López *et al.*, 2011; Güner *et al.*, 2019), and antiaging/antiwrinkle (Andre *et al.*, 2003) properties.

1.4.2.3. Cultivation

There have been several studies on the various potentialities of *Halopteris scoparia*; however, studies on cultivation are scarce. According to Novaczek et al. (1989), *H. scoparia* populations exhibited different growth rates and temperature tolerance. Canadian isolates grew between -2–22 ºC, with optimal growth at 10–15 ºC and died at 22–25 ºC, while European isolates grew better at 10–27 ºC, died at 5 ºC and died at 30–33 ºC after several months.

According to a master's thesis of Silva (2011), *H. scoparia* grows at a wide range of temperatures (10–20 ºC), salinities (20–46 PSU), and two photon flux densities (50 and 150 μmol photons m^{-2} s^{-1}). Silva (2011) obtained the best mean growth rate of 6.8% FW day^{-1} at 0.1 g/L, 15 ºC, 50 μmol photons m^{-2} s^{-1}, and at 12:12 photoperiod. In a master's thesis (Carreiro, 2014) and consequently published in the article by Patarra et al. (2017), it was found that the best amount of biomass and length of *H. scoparia* were recorded at light intensities of 70 and 150 μmol photons m^{-2} s^{-1}. Patarra et al. (2017) obtained higher growth rates of around 9% FW day^{-1} at 150 μmol photons m^{-2} s^{-1}, 2 g/L, photoperiod of 12:12, and at 18 and 24 °C. However, considered that the best overall growth performance (between RGR and productivity) was obtained at 70 μmol photons m^{-2} s^{-1}, 18 ºC, reaching 4.6% day^{-1} (RGR), and biomass output nearing 23 g m^{-3} day^{-1}, since explains that these higher growth values obtained at a higher light intensity may be because of the increase of epiphytes rather than the growth of *H. scoparia*. Knowledge about the ideal conditions for cultivating *H. scoparia* is limited. According to the studies discussed above, it can be concluded that the best growing conditions for *H. scoparia* should be between the density of 0.1 and 2 g/L at 15–18 ºC, 50–70 µmol photons m^{-2} s^{-1}, and a salinity of 20 PSU (Carreiro, 2014; Patarra *et al.*, 2017; Silva, 2011).

Regarding nutrient uptake experiments on *H. scoparia*, the master's thesis Silva (2011), and an abstract proceeding, Silva et al. (2010), *H. scoparia*, cultivated for 14 days with nutrients added daily at a density of 1 g/L, 15°C and at 150 µmol photons m^{-2} s^{-1}, shows that preferentially removed ammonium (NH_4^+), when both nitrogen sources (NO_3^- and NH_4^+) were available. At high concentrations of both nitrogen sources (150 µmol L^{-1}) removal rates exceed 90% with growth rates reaching 3% day^{-1}. However, phosphorus (PO_4^{3-}) removal was less efficient (75–98%). Moreover, Patarra et al. (2016) obtained the best RGR (5.52 ± 1.84% day^{-1}) and biomass production (13.28 ± 4.43 g m^{-2} wk^{-1}) at 2 g/L, independently of the water flow (open flux and semi-closed flux) and removing 3.63 ± 0.47 g m^{-2} day^{-1} of total ammonium nitrogen.

1.5. Objetives of the work

The aim of this research is to evaluate the cultivation potential of *Codium tomentosum* and *Halopteris scoparia* in two different systems. The first experiment consists of evaluating the culture potential of these two species in a controlled environment attached to a substrate and tumble culture at two different densities. This experiment investigates how the culture densities and the cultivation method affect the growth and attachment of these species. The second experiment aims to evaluate the production and nitrogen bioremediation potential of *H. scoparia* in an Integrated Multitrophic Aquaculture (IMTA) system, testing four different culture densities. This experiment investigates how the culture densities affect the growth and productivity of this species and the nitrogen removal rate.

2. Material and Methods

In this work two experiments were carried out. The first experiment was performed at the Estação Piloto de Piscicultura de Olhão (EPPO-IPMA), began in January 2023 and lasted for 8 weeks. The second experiment, was performed at the Piscicultura

Vale da Lama Lda, began in April and lasted for 9 weeks. *Codium tomentosum* was not used in the second experiment since no biomass was available.

2.1. Macroalgae collection and preparation

The macroalgae *H. scoparia* and *C. tomentosum* were collected on the Algarve coast, according to weather conditions and tides (low tide time and height). The date, place, and low tide amplitude are given in Table 2.1. In order to avoid damage to the algae and the environment, the algae were removed from the substrate as gently as possible in order to remove them whole from the substrate where they are attached. Afterward, the algae were placed in net bags and transported in buckets with seawater to the respective locations where the experiments were carried out.

Table 2.1 Macroalgae collection information.

Species	Experiment	Date of collection	Place of collection	Tide height
Halopteris scoparia	1	January 9th	Arrifes Beach	1 m
	2	March 23rd		1.5 m
Codium tomentosum	1	January 23rd	Olhos de Água Beach	1.1 m

After the algae were collected in the first experiment, they were kept indoors, in buckets under artificial light and with aeration for a maximum of 7 days for *H. scoparia* and for 3 days for *C. tomentosum*. During this time, they were cleaned by hand, using tweezers and seawater, previously UVed and filtered, to remove sediment and attached organisms. For the second experiment, the algae were kept in outdoor 110 L tanks for 9 days with aeration, while they were cleaned by hand, removing as many epiphytes as possible.

2.2. Experiment 1 - Cultivation in laboratory

In this experiment, *H. scoparia* and *C. tomentosum* were cultivated under controlled environmental conditions, without (tumble culture) and with substrate (2 mm twisted nylon twine), at two initial culture densities, each with three replicates. The information about the densities used, start dates, and cultivation periods is described in Table 2.2, for each species and cultivation method.

Table 2.2 Information on the methods, species, densities used, and periods of cultivation.

Specie	Cultivation method	Densities	Cultivation started	Period
Halopteris scoparia	Tumble culture	2 g/L and 4 g/L	January 16th	6 weeks
	With substrate	2 g/L and 4 g/L	January 17th	
Codium tomentosum	Tumble culture	4 g/L and 8 g/L	January 25th	5 weeks
	With substrate	33 g/L and 65 g/L	January 26th	

2.2.1. Experiment preparation and cultivation conditions

Before the algae were collected, the whole system was planned. First, it was defined how the containers would be positioned inside the aquariums (maximum of 9 containers per aquarium). Then, all the material was prepared and cleaned (spools, flasks, aquariums, aeration system, culture chamber).

For both cultivation methods, the algae were grown in 500 mL glass containers, properly labeled with the acronym of the current experiment, and covered with parafilm. Each container was subjected to gentle and constant aeration through tubes placed at the base of the container (Figure 2.1). The glass containers used were beakers for all culture methods except for the *H. scoparia* culture for the tumble culture method, where glass bottles were used. The spools were prepared by winding into PVC tubes 3.3 m of previously boiled twine to remove any chemicals that may affect algae attachment. Three small lengths of twine (each with 0.16 m) were added to the main twine to use for sampling purposes and observation under the stereo microscope to quantify growth. In order to keep the spools clean, they were stored in a closed plastic bag until the experiment.

Finally, the temperature, light intensity, and photoperiod were defined. According to other studies, the cultivation conditions chosen were a temperature of 18 °C, light intensity between 70–100 μmol photons m^{-2} s^{-1}, and a photoperiod of 12:12 (Carreiro, 2014; Kang *et al.*, 2008; Patarra *et al.*, 2016).

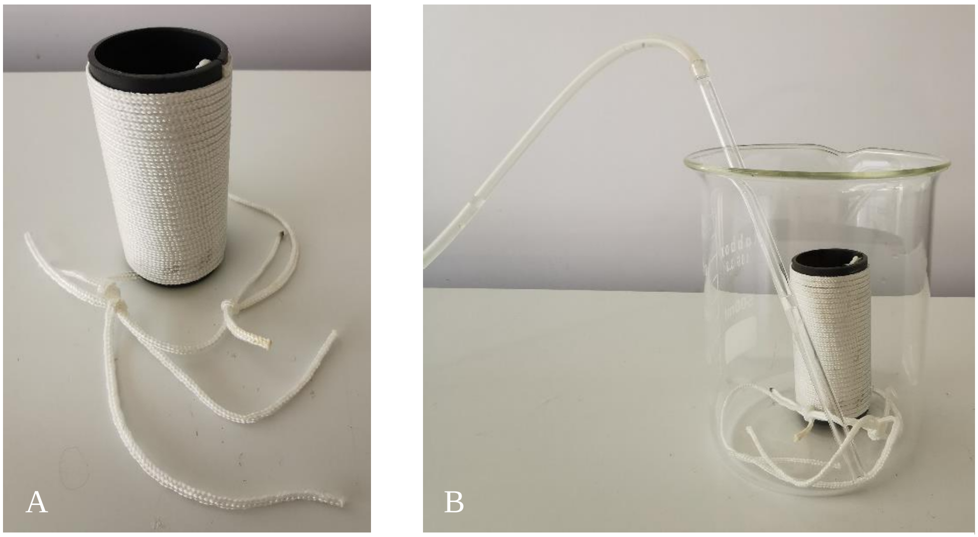

Figure 2.1 A) spool photo; B) aerating system photo.

2.2.2. Macroalgae preparation

The biomass with the lowest number of epiphytes was chosen. Before starting the cultivation, several macroalgae baths were carried out. Individually, the algae were removed from their respective containers and placed in a filter. This filter was placed for 30 seconds in each of the following containers containing: freshwater, followed by autoclaved seawater (salinity 35), autoclaved seawater (salinity 35) with 10% betadine, and three more containers with autoclaved seawater (salinity 35) in order to remove well the betadine from the macroalgae. The freshwater bath was done in order to try to reduce the organisms present in the macroalgae. The betadine bath (10%) was done to try to reduce the amount of epiphytes, according to Carreiro (2014). After the baths, the algae were placed in containers, properly labeled, which were used for the cultivation according to the method tested. Finally, the culture medium was prepared with autoclaved seawater and the addition of GeO_2 (1g/L solution) and the F/2 nutrient both at a rate of 1 ml/L. This medium was placed in each container with the macroalgae, which were then transferred to the cultivation chamber. The GeO_2 was added in order to inhibit the growth of diatoms (Kawai *et al.*, 2005; Lewin, 1966).

2.2.2.1. Tumble cultivation

For tumble cultivation, both species of macroalgae were cut into pieces of around 1 cm before the cleaning baths described above. Then, this biomass was weighed according to the respective density and distributed the containers for cultivation (2 g/L and 4 g/L for *H. scoparia* and 4 g/L and 8 g/L for *C. tomentosum*, both in triplicate), properly labeled for each species.

2.2.2.2. Substrate cultivation

Initially, *H. scoparia* was observed under the microscope, where the existence of reproductive structures was seen and identified as sporangia. After preparing the *H. scoparia* biomass as described above, the cut pieces were again observed under a stereoscopic microscope, where photographs were taken. Subsequently, the pieces were left on wet paper and placed at 4°C overnight. The cold and dehydration were done to provide a thermal shock and promote the release of spores. The following day (after about 13 hours), the biomass was rehydrated, the medium was prepared as described above, placed in the culture beakers with the spool at the target densities of algae, and moved into the culture chamber.

Following the method of Hwang et al. (2008), for *Codium tomentosum*, the macroalgae was weighed (55 g), and the cleaning procedure was performed as described before. Then the macroalga was placed in 150 mL of autoclaved salt water (salinity 35), and the macroalga was blendedfor about 5 min. This mixture resulted in a solution with a density of 367 g/L. This solution was then divided in two and diluted to obtain 550 mL at densities of 65 g/L and 33 g/L. Then 3 replicate spools were placed in each of the solutions for 5 min. After 5 min, a greener coloration was noticed only in the twine that was deeper in the solution, meaning that the algae solution ended up having two densities. Therefore, the spools were placed back in the flasks for more 5 minutes, with aeration, so that the solution was always moving. After these 5 minutes, the spools were left on labeled petri dishes for 2 hours to dry so that the algae could cling to the string. After the 2 hours, the culture medium was prepared as described above and added to the beakers, and the spools were gently placed in their respective beakers and moved into the culture chamber.

2.2.3. Cultivation and Sampling

Once a week, for both cultivation methods and both species, the beakers were cleaned, the medium was renewed, and the biomass weight was recorded. The pH

(Fisherbrand accumet AB150 pH benchtop meter) and salinity (refractometer) were measured in all the beakers before and after the medium was renewed.

2.2.3.1. Tumble culture cultivation

For the tumble culture, for both algae species, the biomass was drained, weighed, and fresh weight was noted. The initial culture density was put back into the containers. Photographs were taken of this initial biomass, about 60 pieces for *H. scoparia* and the entire biomass for *C. tomentosum*, on an A4 piece of paper along with a ruler, and 15 pieces of *H. scoparia* and 6 of *C. tomentosum* under a stereo microscope. The pictures under a stereo microscope were compiled, and two tables were created (Annex 1, and 2 for *C. tomentosum* and *H. scoparia*, respectively).

2.2.3.2. Substrate Cultivation

For the substrate culture method, only for the species *H. scoparia*, pictures were taken weekly of 15 pieces of algae under the stereo microscope to analyze the condition of the macroalgae and to observe whether sporangia are still present (Figure 2.2). When the sporangia became empty (second week), the pieces of algae were removed. For both species, *H. scoparia* and *C. tomentosum*, the three extra spool twines were removed weekly and analyzed under a stereo microscope. Pictures were taken of both sides of the twines in order to ensure uniform sampling and determine if any macroalgae had attached and begun to grow. In the case of *C. tomentosum*, some dark green dots were observed attached to the twine, which subsequently were counted. All these pictures were compiled, and three tables were created (Annex 3, 4, and 5; for *C. tomentosum* twine, *H. scoparia* macroalgae, and *H. scoparia* twine, respectively) to show the evolution of the cultivations from the date of the start to the final date of cultivation.

Figure 2.2 Photo of *H. scoparia* under the stereo microscope with reproductive structures.

2.3. Experiment 2 – Cultivation in IMTA

In this experiment, *Halopteris scoparia* was cultivated in an IMTA system at four initial culture densities (5 g/L, 10 g/L, 15 g/L, and 20 g/L), each with 3 replicates. Cultivation started on April 1st and lasted for 9 weeks.

2.3.1. Experimental setup

Twelve filters and 12 buckets (Figure 2.3) were prepared for cultivating the macroalgae. Each bucket contained a clear hose attached with silicone at the bottom of the bucket for aeration and a hole at the top of the bucket where a 12 cm PVC tube was attached, also with silicone. This tube had the function of placing the filter so that the algae would not get out. For the filters, PVC tubes and netting (0.5 mm mesh size) were used. Two pumps (water and air) were set up, and the water and air were directed through tubes to the table where the twelve buckets were positioned. These tubes were fixed to the top structure of the table, and from each main tube, twelve tubes came out (Figure 2.4). The air tubes were connected to a tube that was fixed to the bottom of the bucket (Figure 2.3). This tube was pierced at regular intervals to keep the algae in constant motion. This optimized the macroalgae exposure to light and nutrients present in the water (Abreu *et al.*, 2011). An earthen pond with fish (*Diplodus sargus* and *Sparus aurata*) provided the wastewater for the system. The macroalgae initial biomass was cleaned as previously mentioned (point 2.1) and then weighed. The stocking densities were 5 ± 0.15 g/L, 10 ± 0.15 g/L, 15 ± 0.15 g/L, and 20 ± 2 g/L.

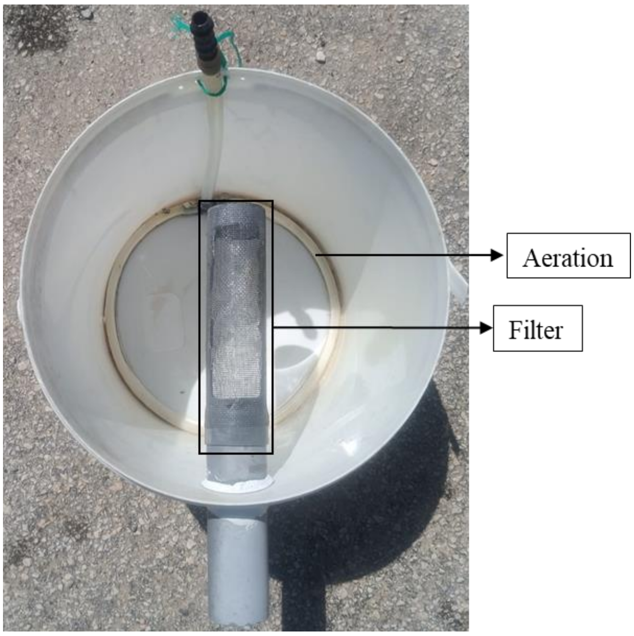

Figure 2.3 Photo of the macroalgae cultivation tanks.

Figure 2.4 Photo of the macroalgae cultivation system.

2.3.2. Cultivation and Sampling

Twice daily (morning and afternoon), the water flow was adjusted manually for each tank at 1.2 l/min (72 L/h, 4 volumes/hour), and water quality parameters were measured, namely temperature (HANNA Checktemp Dip), dissolved oxygen (OxyGuard Handy Polaris 2), pH (WTW Multi 3430 SET F), and salinity (portable refractometer).

Water samples from the inlet water were collected, filtered (MCA Cellulose Acetate Membrane Filter, 0.45 µm), and frozen to be sent to an external laboratory for analysis of nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$), ammonium ($N\text{-}NH_4^+$), and phosphate ($P\text{-}PO_4^{3-}$) by Segmented Flow Analyzers (SFA) in MARINNOVA. These samples were taken every two days at the same time (18 h) during the experiment and four times (throughout the day) every two weeks to analyze the variation of the concentration throughout the day. On days when water samples were taken four times a day, measurements of water quality parameters (pH, temperature, dissolved oxygen, and salinity) were also taken to analyze how these vary throughout the day.

Once a week, the macroalgae biomass was collected. The excess superficial water was drained by hand, and the biomass was weighed and recorded in fresh weight (FW) to calculate the specific growth weight (SGR) and yield (Y). Then the density was reduced back to the initial biomass while the tanks were cleaned. The biomass required to achieve the target densities was then weighed and set aside while the tanks were cleaned. After, the wastewater supply was re-started, and the biomass was placed into the clean tanks. To minimize the positional influence, the macroalgae were placed in randomly chosen tanks each week.

During the weekly sampling, samples of *H. scoparia* were collected from all replicas at the beginning (April 5th), middle (April 30th), and end (June 4th) of cultivation. These samples were all weighed, dried in an oven (60 ºC) for three days until no weight change was observed, macerated until a fine powder was obtained using a mortar and pestle, and placed in Eppendorf's tubes, which were stored in a desiccator until analysis of the carbon (C) and nitrogen (N) content of the macroalgae biomass by Organic Elemental Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS) in MARINNOVA. These samples were also used in the determination of dry weight (DW) yield calculations.

2.4. Data analysis

2.4.1. Growth and yield calculation

The specific growth rate (SGR) and yield (Y) were calculated. The SGR was calculated based on the formula in equation 1 (% FW d^{-1}). According to Yong et al. (2013), this formula is the most accurate of all those available. The biomass yield (Y)

(Abreu *et al.*, 2011) was calculated differently, depending on the experiment. For the first experiment, it was calculated with equation 2 ($g\ FW\ L\ wk^{-1}$), and for the second experiment with equations 3 ($g\ FW\ m^2\ wk^{-1}$) and 4 ($g\ DW\ m^2\ wk^{-1}$). The N-yield by *H. scoparia* biomass production was calculated by multiplying the biomass yield ($g\ DW\ m^2\ wk^{-1}$) from equation 4 by the nitrogen content of *H. scoparia* (equation 4).

$$SGR\ (\%\ FW\ d^{-1}) = \left[(\omega_t\ /\ w_0)^{1/t} - 1\right] \times\ 100 \text{ (equation 1)}$$

$$Y\ (g\ FW\ l\ wk^{-1}) = [(\omega_t - w_0)\ L]\ /\ t \text{ (equation 2)}$$

$$Y\ (g\ FW\ m^2\ wk^{-1}) = [(\omega_t - w_0)\ /\ A]\ /\ t \text{ (equation 3)}$$

$$Y\ (g\ DW\ m^2\ wk^{-1}) = \left[\left((\omega_t - w_0)\right)DW/FW\ /\ A\right]\ /\ t \text{ (equation 4)}$$

$$N\!-yield = Y\ \times NC\ \text{ (equation 5)}$$

Where, FW = fresh weight; DW = dry weight; W_0 = initial biomass (g FW); W_t = final biomass (g FW); t = total of culture days; wk = week; L = liters; A = m^2 area of surface cultivation tank; DW/FW is the dry weight/fresh weight ratio; NC = nitrogen content (%).

2.4.2. Statistical analysis

The statistical analyses were performed using the software RStudio (R version 4.1.1) and a significance level (α) of 0.05. When p was lower than α, statistical differences were identified. First, normality (Shapiro-Wilk's test) and variance homogeneity (Levene's test) were tested. When both assumptions were fulfilled, the parametric test, T-test, or ANOVA were used to test for differences between means of the data (two or more samples). If the assumptions were not fulfilled, data transformation was attempted (natural logarithmic). When the transformed data fulfilled the presumptions, parametric tests were used. Otherwise, the nonparametric Kruskal-Wallis test was used. A post-hoc test was carried out to analyze the significant differences or interactions that these statistical tests revealed. The Tukey test was applied after the ANOVA test, while the Dunn-Bonferroni test followed the Kruskal-Wallis test.

These tests were done in order to analyze if the SGR, yield, and length varied significantly between the stocking densities and throughout the weeks for both species in the tumble cultivation. For the IMTA system, the environmental conditions were analyzed to see if there were variations in oxygen and pH between the stoking densities (5, 10, 15, and 20 g/L) for each period of the day (morning and afternoon). It was also analyzed if there were differences in the quantity of nutrients ($P\text{-}PO_4^{3-}$; $N\text{-}NH_4^+$; $N\text{-}NO_3^-$; and $N\text{-}NO_2^-$) present in the water throughout the weeks (first to ninth weeks) and throughout the day (9h, 12h, 15h, and 18h). Regarding the macroalgae, it was analyzed if the SGR (% FW d^{-1}), yield (g FW L wk^{-1}), and N-yield (g DW N m^{-2} wk^{-1}) differed according to the different stocking densities (5, 10, 15, and 20 g/L) and differed throughout the weeks (from the first to ninth week). Furthermore, it was analyzed whether the content of nitrogen (%N), carbon (%C), and C:N ratio was significantly different between the different cultivation densities (5, 10, 15, and 20 g/L) and over time (weeks 0, 4, and 9).

3. Results

3.1. Experiment 1 – Cultivation in laboratory

3.1.1. Tumble culture method

3.1.1.1. Environmental conditions

The initial pH and salinity values (for each week), the mean value ± standard deviation, and the minimum and maximum values (after each week of cultivation) are described in the tables 3.1 and 3.2, respectively, for *C. tomentosum* and *H. scoparia.* Evaporation of the culture medium was observed in the weekly samplings for both species. Accordingly, the final salinity and pH values after each week of cultivation were higher than the initial values for both species. Except at the third and fifth weeks for *C. tomentosum* and the fifth week for *H. scoparia*, when the mean value of pH decreased, both for the higher density of cultivation. The pH values for *C. tomentosum* ranged from 8.2 (both densities) to 8.8 (8 g/L) and 8.9 (4 g/L); for *Halopteris scoparia*, it ranged from 8.2 to 9.1 (4 g/L) and from 8.3 to 9.3 (2 g/L). Regarding the salinity, for *C. tomentosum*, it varied between 34 (both densities) to 40 (4 g/L) and 45 (8 g/L), while for *H. scoparia*, it ranged from 35 (both densities) to 56 (2 g/L) and 62 (4 g/L).

Table 3.1 pH and salinity values of *C. tomentosum* cultivation in tumble culture.

		pH			Salinity		
Week	**Treatment**	**Initial**	**Final**	**Min – Max**	**Initial**	**Final**	**Min – Max**
1	**4 g/L**	-	8.4 ± 0.2	8.2 - 8.6	-	37.7 ± 2.3	35.0 - 39.0
	8 g/L		8.5 ± 0.1	8.4 - 8.6		37.7 ± 2.9	36.0 - 41.0
2	**4 g/L**	8.4	8.5 ± 0.1	8.4 - 8.6	35	38.3 ± 2.9	35.0 - 40.0
	8 g/L		8.6 ± 0.2	8.4 - 8.8		36.7 ± 3.1	34.0 - 40.0
3	**4 g/L**	8.5	8.7 ± 0.2	8.6 - 8.9	34	35.3 ± 1.5	34.0 - 37.0
	8 g/L		8.4 ± 0.0	8.4		41.0 ± 4.6	36.0 - 45.0
4	**4 g/L**	8.3	8.4 ± 0.2	8.3 - 8.6	33	35.7 ± 0.6	35.0 - 36.0
	8 g/L		8.4 ± 0.1	8.3 - 8.4		36.0 ± 2.0	34.0 - 38.0
5	**4 g/L**	8.5	8.5 ± 0.2	8.3 - 8.6	33	36.0 ± 1.7	35.0 - 38.0
	8 g/L		8.3 ± 0.2	8.2 - 8.5		38.0 ± 1.7	37.0 - 40.0

Table 3.2 pH and salinity values of *H. scoparia* cultivation in tumble culture.

		pH			Salinity		
Week	**Treatment**	**Initial**	**Final**	**Min – Max**	**Initial**	**Final**	**Min – Max**
1	**2 g/L**	-	8.7 ± 0.3	8.4 - 9.0	-	47.7 ± 8.0	40.0 - 56.0
	4 g/L		8.7 ± 0.3	8.4 - 9.0		54.7 ± 7.0	48.0 - 62.0
2	**2 g/L**	-	8.4 ± 0.0	8.4	-	38.0 ± 1.7	37.0 - 40.0
	4 g/L		8.2 ± 0.0	8.2		39.7 ± 1.5	38.0 - 41.0
3	**2 g/L**	8.3	8.4 ± 0.1	8.4 - 8.5	34	39.0 ± 3.6	35.0 - 42.0
	4 g/L		8.3 ± 0.2	8.2 - 8.5		41.0 ± 4.0	37.0 - 45.0
4	**2 g/L**	8.3	8.5 ± 0.1	8.4 - 8.5	34	37.7 ± 3.1	35.0 - 41.0
	4 g/L		8.6 ± 0.4	8.3 - 9.0		37.3 ± 2.1	35.0 - 39.0
5	**2 g/L**	8.6	8.6 ± 0.6	8.3 - 9.3	34	39.3 ± 5.1	35.0 - 45.0
	4 g/L		8.4 ± 0.1	8.3 - 8.4		38.3 ± 1.5	37.0 - 40.0
6	**2 g/L**	8.1	8.4 ± 0.2	8.3 - 8.6	33	39.3 ± 2.5	37.0 - 42.0
	4 g/L		8.6 ± 0.4	8.3 - 9.1		38.7 ± 4.7	35.0 - 44.0

3.1.1.2. *Codium tomentosum* growth

The specific growth rate (SGR) of *Codium tomentosum* (Figure 3.1) shows that both densities grow throughout each week (except in the fifth week for 8 g/L). The highest mean SGR (% FW d^{-1}) values were recorded at a density of 4 g/L and were 10.61 ± 3.70

(fifth week) and 10.01 ± 0.22 (fourth week). At a density of 8 g/L, the highest mean SGR (% FW d^{-1}) values were 6.58 ± 0.69 (fourth week) and 5.78 ± 0.78 (fifth week).

An independent t-test revealed that the SGR at 4 g/L was significantly higher than at 8 g/L on the third (9.68 ± 0.499) and fourth (10.01 ± 0.22) weeks (both p = 0.001). Nevertheless, density did not have a significant effect on the SGR in the other weeks of cultivation (p > 0.05). For each density, it was tested if the SGR differed throughout weeks (time). For 4 g/L, a Kruskal-Wallis test and for 8 g/L, a one-way ANOVA showed significant differences (p = 0.025 and p < 0.001, respectively). Consecutively, the Dunn-Bonferroni post-hoc test (4 g/L) and the Tukey post-hoc test (8 g/L) showed where there are significant differences, represented in Figure 3.1.

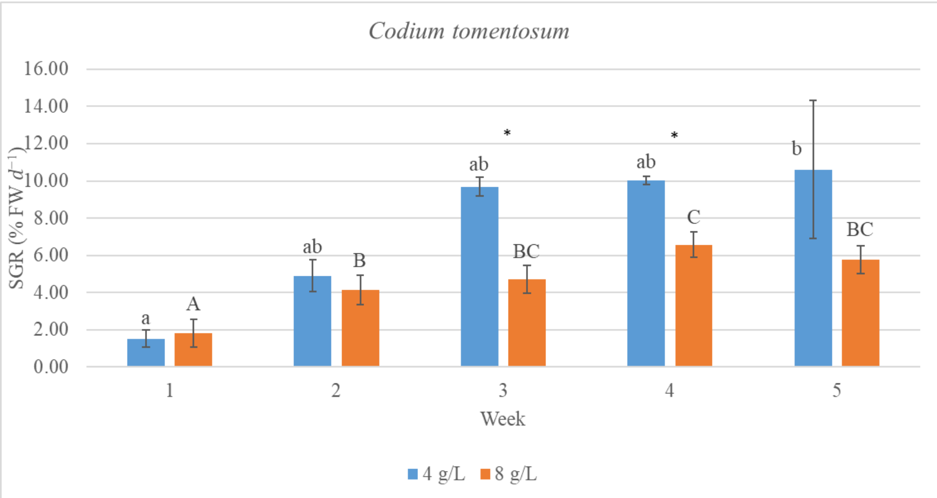

Figure 3.1 Mean of specific growth rate throughout the weeks of *C. tomentosum* (n = 3). The bars represent the mean values of SGR for each density (4 and 8 g/L). Means with an asterisk differ significantly (p ≤ 0.05) between densities within each week. Means sharing a letter do not differ significantly (p > 0.05) between the five weeks, with lowercase letters for 4 g/L and uppercase letters for 8 g/L.

The yield of *C. tomentosum* (Figure 3.2) appears to have an increasing tendency over the weeks, except for the fifth week at a density of 8 g/L. The highest mean values of yield (g FW L wk^{-1}) registered were 4.57 ± 0.65 (8 g/L, week 4) and 4.38 ± 2.02 (4 g/L, week 5). According to a one-way ANOVA test, the different densities had no significant effect on the yield (p > 0.05). However, the Kruskal-Wallis (4 g/L) and a one-way ANOVA test (8 g/L) showed that different weeks had a significant effect on yield (p = 0.026 and p = 0.001, respectively). Following, the Dunn-Bonferroni post-hoc test (4 g/L) and the Tukey post-hoc test (8 g/L) showed where there are significant differences, represented in Figure 3.2.

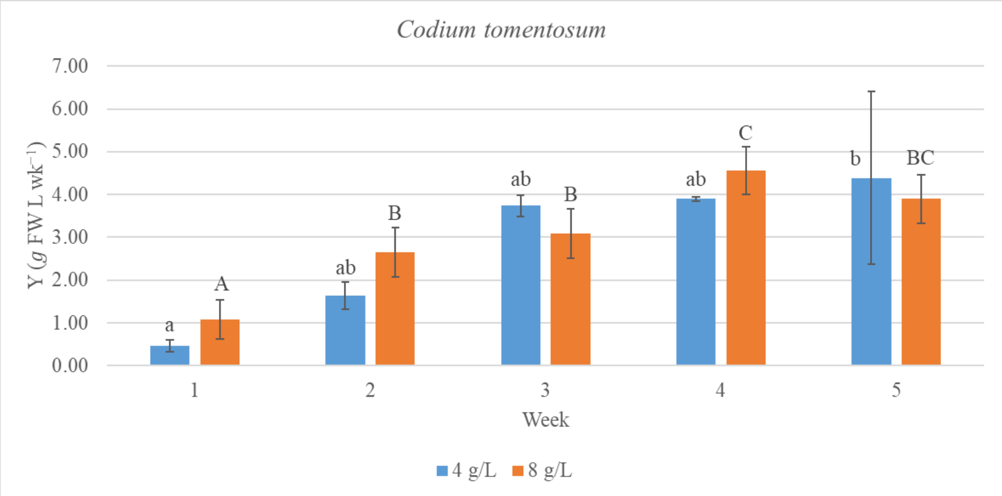

Figure 3.2 Mean of yield throughout the weeks of *C. tomentosum* (n = 3). The bars represent the mean values of yield for each density (4 and 8 g/L). Means sharing a letter do not differ significantly ($p > 0.05$) between the five weeks, with lowercase letters for 4 g/L and uppercase letters for 8 g/L.

The length of *Codium tomentosum*, represented in Figure 3.3, shows that there was consistent but small growth for both densities. The highest mean lengths were registered in the fifth week: 1.48 cm ± 0.24 (4 g/L) and 1.48 cm ± 0.22 (8 g/L). To analyze if there were statistical differences in the lengths of *C. tomentosum* between the two densities, a Kruskal-Wallis test was performed for the initial biomass, first, second, and third weeks, and a one-way ANOVA was performed for the fourth and fifth weeks. According to these tests, only the initial length at the density of 4 g/L (1.19 ± 0.20) showed to be significantly higher than at the density of 8 g/L (1.12 ± 0.20) ($p < 0.001$). In addition, a Kuskal-Wallis test showed that, for both densities, there are significant differences (represented in Figure 3.3) in lengths over the weeks (both with $p < 0.001$). Nevertheless, it is relevant to mention that the Dunn-Bonferroni post-hoc test showed that, at both densities, the length is significantly lower at the initial biomass and first week than at the third, fourth, and fifth weeks ($p \leq 0.05$).

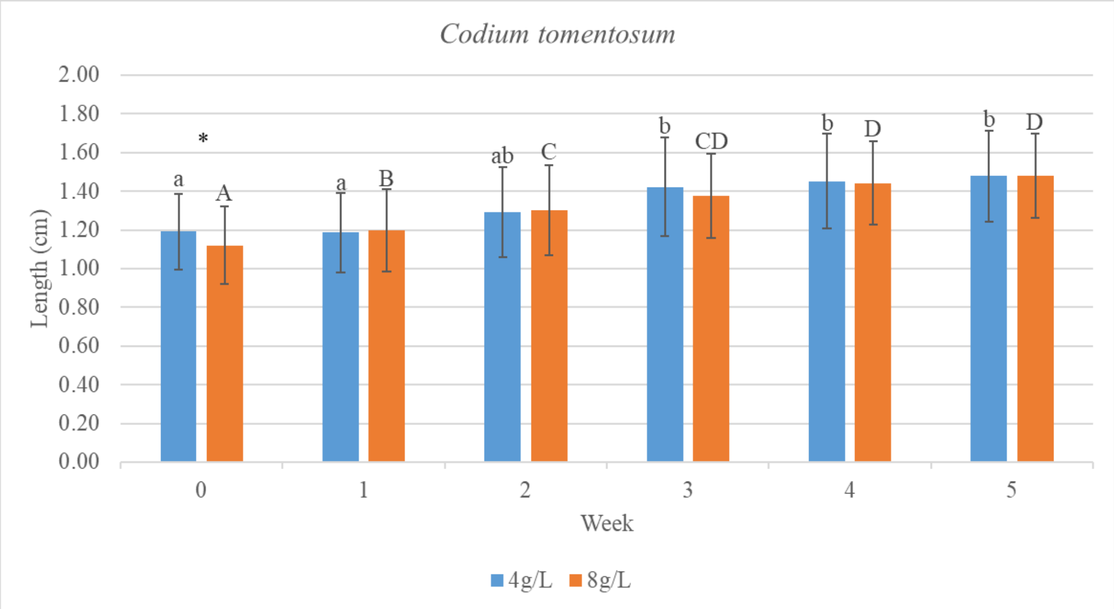

Figure 3.3 Mean of lengths throughout the weeks of *C. tomentosum*. The bars represent the mean values of lengths for each density (4 and 8 g/L). Means with an asterisk differ significantly ($p \leq 0.05$) between densities for each week. Means sharing a letter do not differ significantly ($p > 0.05$) between the five weeks, with lowercase letters for 4 g/L and uppercase letters for 8 g/L Since the initial stocking density was set at each sampling, the n value dropped over the weeks. Accordingly, for weeks 0 through 5, for the density of 4 g/L, n = 63, 59, 44, 33, 24, 23; and for the density of 8 g/L, n = 117, 107, 80, 61, 44, 43

In order to visually demonstrate the development of *C. tomentosum* over the 5 weeks of cultivation, a table with 3 photos per replicate is shown in Annex 1. The macroalgae condition before cultivation is shown in the first line of the table (25/01/2023). At this time the macroalgae had a dark green color and some red contamination. After two weeks of cultivation, there has been a large increase in contamination by green algae and a slightly brownish coloration of the *C. tomentosum*. The contamination continues to increase, and the *C. tomentosum* continues to deteriorate in the third, fourth, and fifth weeks. Although the lowest density (4 g/L) of *C. tomentosum* continues to grow, it does not have the color it had at the start of cultivation.

3.1.1.3. *Halopteris scoparia* growth

The specific growth rate (SGR) of *Halopteris scoparia* (Figure 3.4) shows that macroalgae at both densities grew throughout each week, except for the density of 2 g/L on the fifth week. The highest mean values of SGR (% FW d^{-1}) registered were 16.07 ± 1.32 (sixth week) and 12.45 ± 1.79 (fourth week), both at the density of 2 g/L. Whereas for the density of 4 g/L, the highest mean values of SGR (% FW d^{-1}) recorded were lower, 8.84 ± 1.21 (sixth week) and 8.83 ± 1.27 (fifth week).

According to a one-way ANOVA test, the density of 2 g/L has a significantly higher SGR than 4 g/L in the second and fourth weeks (both with $p < 0.001$), in the third week ($p = 0.017$), and in the fifth and sixth weeks (with $p = 0.018$ and $p = 0.002$, respectively). Moreover, a one-way ANOVA was carried out for each density and showed that, for both densities, there are statistical differences over the weeks ($p < 0.001$). Therefore, the Tukey post-hoc test was conducted, and the significant differences ($p \leq 0.05$) are shown in Figure 3.4.

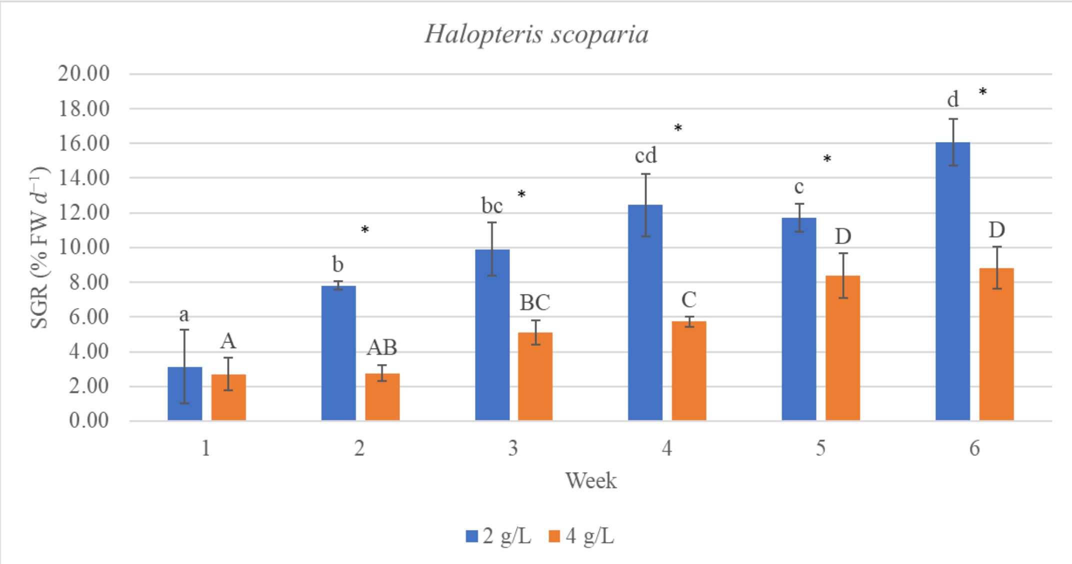

Figure 3.4 Mean of specific growth rate throughout the weeks of *H. scoparia* (n = 3). The bars represent the mean values of SGR for each density (2 and 4 g/L). Means with an asterisk differ significantly ($p \leq 0.05$) between densities within each week. Means sharing a letter do not differ significantly ($p > 0.05$) between the six weeks, with lowercase letters for 2 g/L and uppercase letters for 4 g/L.

The yield of *H. scoparia* (Figure 3.5) appears to have an increasing tendency over the weeks, except for the second week at a density of 4 g/L and for the fifth week at a density of 2 g/L. The highest mean values of yield (g FW L wk^{-1}) registered were 3.75 ± 0.45 (2 g/L) and 3.26 ± 0.57 (4 g/L), both in the sixth week.

A two-way ANOVA test was performed to analyze if the yield differed according to the different stocking densities and if it differed along weeks (time). According to this test, the time and the interaction between the two factors had a significant effect on yield

($p < 0.001$ and $p = 0.023$, respectively). The significant differences between the weeks were represented in Figure 3.5. Moreover, it is important to mention that for both densities, the yield in the first week is significantly lower than in the fourth, fifth, and sixth weeks (with for 2 g/L all $p < 0.001$ and for 4 g/L $p = 0.043$; $p < 0.01$ and $p = 0.01$, respectively); in the second week it is significantly lower than in the fourth and sixth weeks (with for 2 g/L $p = 0.021$ and $p < 0.001$ and for 4 g/L $p = 0.047$ and $p < 0.01$, respectively); and in the third and fourth weeks it is significantly lower than in the sixth week (with $p < 0.01$ and $p = 0.032$ for 2 g/L and $p = 0.001$ and $p = 0.009$ for 4 g/L, respectively). Even though there were no significant differences between the different densities, 2 g/L was the one that, in terms of yield, showed the highest yield values over most of the cultivation time.

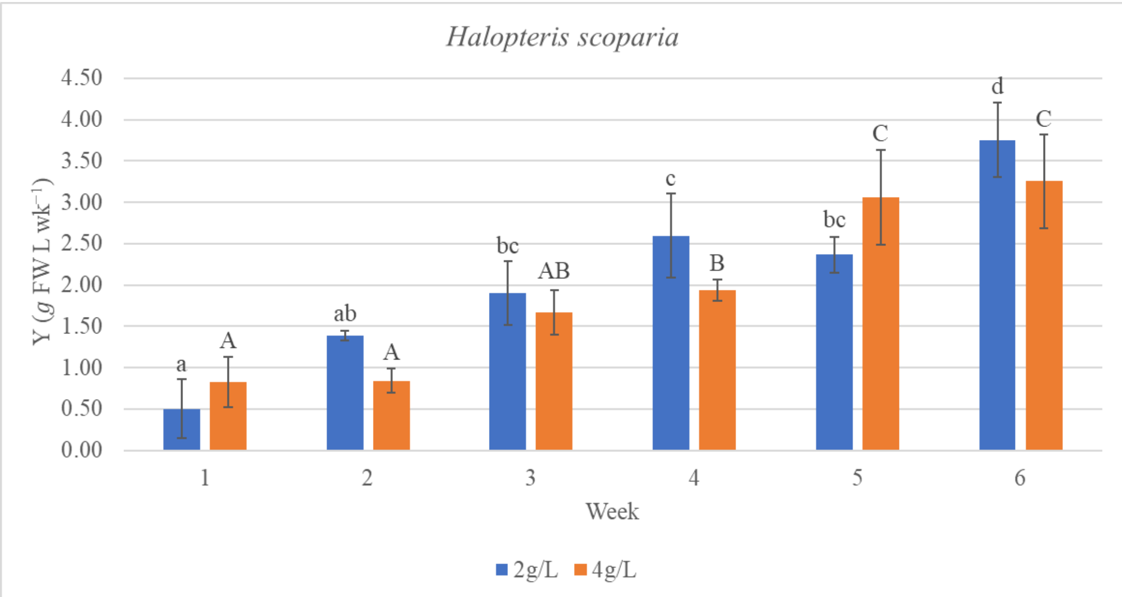

Figure 3.5 Mean of yield throughout the weeks of *H. scoparia* (n = 3). The bars represent the mean values of yield for each density (2 and 4 g/L). Means sharing a letter do not differ significantly ($p > 0.05$) between the six weeks, with lowercase letters for 2 g/L and uppercase letters for 4 g/L.

For the length (cm) of *H. scoparia*, the highest mean values registered were 0.93 ± 0.39 (2 g/L) and 0.93 ± 0.37 (4 g/L), both at the beginning of cultivation. Hereinafter, the highest mean values of lengths (cm) registered were 0.94 ± 0.38 (4 g/L) and 0.85 ± 0.34 (2 g/L), both at the first week. In order to analyze if there were statistical differences in the lengths of *H. scoparia* between the two densities for all weeks, a Kruskal-Wallis test was performed. According to this test, overall, the 4 g/L density showed significantly higher length than 2 g/L density at the first ($p = 0.05$), fourth ($p = 0.02$), fifth, and sixth (both $p < 0.001$) weeks, as shown in figure 3.6.

In addition, for each density, a Kuskal-Wallis test was performed; accordingly to this test, the different weeks had a significant effect on lengths ($p < 0.001$). Afterward, the Dunn-Bonferroni post-hoc test was performed and demonstrated where the significant differences are, as can be observed in Figure 3.6. Nevertheless, it is relevant to mention that for both densities, the length is significantly higher at the beginning of the cultivation (initial biomass and first week) than the other weeks. Also, for a density of 2 g/L, it is possible to observe that the length of the algae decreases over the weeks. Furthermore, at a density of 4 g/L, at the end of the experiment, the length of the algae was shorter than at the beginning. However, there was not always a continuous decrease in length, like at the density of 2 g/L. In the fourth, fifth, and sixth weeks, the length of the macroalgae at 4 g/L was higher than in the third week but not higher than the initial length.

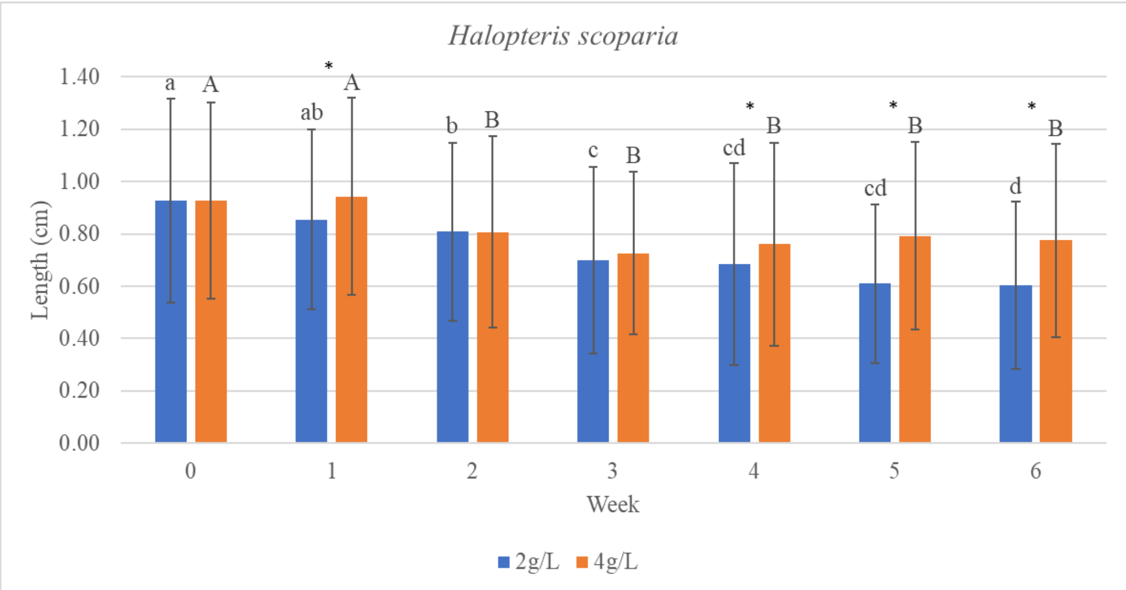

Figure 3.6 Mean of lengths throughout the weeks of *H. scoparia*. The bars represent the mean values of lengths for each density (2 and 4 g/L). Means with an asterisk differ significantly ($p \leq 0.05$) between densities within week. Means sharing a letter do not differ significantly ($p > 0.05$) between the six weeks, with lowercase letters for 2 g/L and uppercase letters for 4 g/L. Accordingly, for weeks 0 through 6, for the density of 2 g/L, n = 231, 212, 194, 195, 184, 193; 194 and for the density of 4 g/L, n = 206, 209, 250, 196, 201, 204, 212.

In Annex 2, a table is presented with three photos per replicate of the pieces of the macroalga *H. scoparia* over the 6 weeks of cultivation to visually demonstrate their development. Before cultivation began, photographs were taken of the pieces of algae to document the evolution of the appearance of the macroalgae throughout the cultivation.

At this point, the macroalgae had separated, branched filaments, a bright dark brown color, and showed that it was already growing, as the tips were a lighter brown (shown in the second photo of all the replicates at both densities in Annex 2). In addition, it already exhibited contamination from other macroalgae (visible in the third photo, R3 at 2 g/L, and in the third photo of all the replicates, R1, R2, and R3 at 4 g/L in Annex 2). After two weeks of cultivation, it was observed that the macroalgae continued to grow and that there had been an increase in contamination, especially at the lowest density (2 g/L). After three weeks of cultivation, it was noted that the contamination had increased in both densities and that the branched filaments of *H. scoparia* are no longer well-defined and separate and have started to appear bundled by a light brown "mass". This was evident in the second and third photos, R1 and R2 at 2 g/L; the first photo, R3 at 2 g/L; and in the first and second photos of all the replicates, R1, R2, and R3 at 4 g/L. From the third week of cultivation until the sixth, the changes observed were an increase in the alterations recorded in the third week of cultivation.

3.1.2. Substrate method

3.1.2.1. Environmental conditions

Tables 3.3 and 3.4, respectively, for *C. tomentosum* and *H. scoparia*, provide information of the initial pH and salinity values (for each week), the mean value ± standard deviation, and the minimum and maximum values (after each week of cultivation). Evaporation of the culture medium was observed in the weekly samples for both species. Accordingly, the final salinity and pH values after each week of cultivation were higher than the initial values for both species. Except for the first and fifth weeks, when the mean value of pH decreased for *C. tomentosum* and *H. scoparia*, respectively, both for the two densities. Salinity also showed an exception when it decreased in the third week at the lowest density of *H. scoparia*. Concerning the pH values, for *C. tomentosum*, it ranged from 8.0–9.8 (4 g/L) and 8.1–9.5 (8 g/L); for *H. scoparia, it* ranged from 8.1–9.4 (2 g/L) and 8.1–9.0 (4 g/L). Regarding the salinity, for *C. tomentosum*, it varied between 34–46 (4 g/L) and 34–64 (8 g/L), while for *H. scoparia, it* ranged from 34–100 (2 g/L) and 34–57 (4 g/L).

Table 3.3 pH and salinity values of *C. tomentosum* cultivation in substrate method.

		pH			Salinity		
Week	**Treatment**	**Initial**	**Final**	**Min – Max**	**Initial**	**Final**	**Min – Max**
1	**4 g/L**	8.5	8.0 ± 0.1	8.0 - 8.1	34	34.7 ± 0.6	34.0 - 35.0
	8 g/L		8.1 ± 0.0	8.1		36.3 ± 1.5	35.0 - 38.0
2	**4 g/L**	8.3	8.4 ± 0.1	8.3 - 8.5	35	36.7 ± 2.9	35.0 - 40.0
	8 g/L		8.5 ± 0.3	8.3 - 8.8		35.7 ± 1.2	35.0 - 37.0
3	**4 g/L**	8.3	8.7 ± 0.2	8.5 - 8.9	35	38.7 ± 4.6	36.0 - 44.0
	8 g/L		8.6 ± 0.1	8.6 - 8.7		37.3 ± 3.5	34.0 - 41.0
4	**4 g/L**	8.4	9.0 ± 0.7	8.4 - 9.8	33	35.0 ± 1.7	34.0 - 37.0
	8 g/L		9.3 ± 0.4	8.8 - 9.5		38.0 ± 0.0	38.0
5	**4 g/L**	8.3	8.8 ± 0.3	8.5 - 9.1	33	43.3 ± 3.8	39.0 - 46.0
	8 g/L		8.9 ± 0.3	8.6 - 9.2		49.0 ± 14.1	36.0 - 64.0

Table 3.4 pH and salinity values of *H. scoparia* cultivation in substrate method.

		pH			Salinity		
Week	**Treatment**	**Initial**	**Mean ± SD**	**Min – Max**	**Initial**	**Final**	**Min – Max**
1	**2 g/L**	-	8.2 ± 0.1	8.1 - 8.3	-	78.0 ± 20.3	60.0 - 100.0
	4 g/L		8.5 ± 0.2	8.4 - 8.7		52.3 ± 4.2	49.0 - 57.0
2	**2 g/L**	-	8.5 ± 0.5	8.2 - 9.0	-	82.7 ± 27.2	35.0 - 84.0
	4 g/L		8.2 ± 0.1	8.1 - 8.2		50.0 ± 7.0	42.0 - 55.0
3	**2 g/L**	8.4	9.1 ± 0.3	8.9 - 9.4	35	34.7 ± 0.6	34.0 - 35.0
	4 g/L		8.7 ± 0.3	8.5 - 9.0		36.7 ± 0.6	36.0 - 37.0
4	**2 g/L**	8.3	8.5 ± 0.2	8.3 - 8.7	34	36.3 ± 2.3	35.0 - 39.0
	4 g/L		8.4 ± 0.2	8.3 - 8.6		39.3 ± 5.0	34.0 - 44.0
5	**2 g/L**	8.5	8.3 ± 0.2	8.2 - 8.5	34	38.0 ± 2.6	36.0 - 41.0
	4 g/L		8.3 ± 0.2	8.2 - 8.5		37.3 ± 2.5	35.0 - 40.0
6	**2 g/L**	8.3	8.4 ± 0.1	8.3 - 8.4	33	36.0 ± 2.0	34.0 - 38.0
	4 g/L		8.4 ± 0.0	8.4		41.0 ± 4.6	36.0 - 45.0

3.1.2.2. *Codium tomentosum* growth

In Annex 3, a table is presented with three photographs per replicate of the piece of twine on which *C. tomentosum* would attach and grow throughout five weeks. According to the photographs, it can be assumed that the dark green dots that appeared on the twine in the second week of cultivation are *C. tomentosum* and that it has attached to the twine. Additionally, in the second week of cultivation, there are some green lines on the twine, which appear to be contamination by another green algae (photos of R1 at both densities). Following, in the third week, it is possible to observe that all replicates of both densities show more contamination from other algae. Furthermore, it is possible to observe more dark green dots attached to the twine (as shown in the first photo, R1 at 33 g/L; the first photo of R1, and the first and second photos of R2 and R3, at 65 g/L). Finally, in the fourth and fifth weeks, more dark green dots and contamination by other green algae continue to appear. However, contamination seems to be more prevalent at the lower density (33 g/L).

As previously mentioned, in the second week, it was possible to detect dark green dots on the twines. The mean ± standard deviation and minimum and maximum number of these dark green dots observed in the 12 photos at the two magnifications M3 and M1 (corresponding to 2.87 and 11.04 mm of twine per photo, respectively) are presented in Table 3.5. As it is possible to observe, throughout the week, the number of dots that appeared to be *C. tomentosum* increased at both densities. Additionally, it is important to note that the number of dots visualized was higher at the higher density (65 g/L), demonstrating that a higher concentration of macroalgae is important for successful cultivation.

Table 3. 5 Dark green dots observed (Mean ± standard deviation; [minimum, maximum]) in photographs of the twines of *C. tomentosum* spools.

			Density	
Data	**Week of cultivation**	**Magnification**	**Low (33 g/L)**	**High (65 g/L)**
09/02/2023	**2**		1 ± 0.87; [0, 2]	0.78 ± 0.83; [0, 2]
16/02/2023	**3**	M3	1.78 ± 2.49; [0, 8]	1.89 ± 1.05; [0, 3]
23/02/2023	**4**		1.00 ± 1.00; [0, 3]	2.78 ± 1.20; [1, 4]
02/03/2023	**5**	M1	0.78 ± 0.97; [0, 3]	1.89 ± 1.69; [0, 6]

3.1.2.3. *Halopteris scoparia* growth

In Annexes 4 and 5, tables with three photographs per replicate (R1, R2, and R3) are presented, showing respectively the pieces of macroalgae of *H. scoparia* and the pieces of twine on which *H. scoparia* would attach and grow throughout six weeks. According to the photographs, in annex 5, it cannot be assumed that the *H. scoparia* attached to the twine. At the beginning of the experiment, the pieces of macroalgae present in the medium exhibited clearly defined sporangia, as seen in the Annex 4 in the higher magnification photos, at the first week (16/01/2023), and both densities. Over two weeks of cultivation, the sporangia gradually became less visible and defined, that is, empty (as shown in both densities across all replicates in the third photo). Meanwhile, *H. scoparia* appeared to grow, and contamination increased. At the second week, the macroalgae was removed from the medium since the sporangia was empty.

Regarding the twine, in the second week, contamination by green algae was observed (as shown in all photos of R1 and R2, at both densities), and there were more brownish spots (second photograph of R3 at 4 g/L). From the third week onward, there were more brownish spots and a brownish color on the twines. However, it could not be confirmed that it was *H. scoparia*. Furthermore, the twines showed some dots of green algae that could be contamination by *C. tomentosum*; however, it was not as developed as that observed in its own cultivation (as shown in the fifth week at 4 g/L, R2, third photo; and in the sixth week at 2 g/L, R2, first photo, and at 4 g/L, R2, third photo).

3.2. Experiment 2 – Cultivation in IMTA

3.2.1. Environmental Conditions

The temperature variation during this experiment was registered twice a day (morning and afternoon) and is presented in Figure 3.7. For the values registered in the morning, the minimum values were 14.6 ºC (5 g/L), 16 ºC (10 and 15 g/L), and 16.1 ºC (20 g/L), all registered in the first week. The maximum values were 22.3 ºC (5, 15, and 20 g/L), all recorded in the ninth week, and 22.2 ºC (10 g/L) recorded in the fourth and ninth weeks. In addition, for the values recorded in the afternoon, the minimum values were 17.2 ºC (5, 10, 15 and 20 g/L), recorded during the first week. The maximum values were 25.4 ºC (5, 15 and 20 g/L) and 25.7 ºC (10 g/L), which were all recorded during the fourth week.

The same trend was observed for all densities and both morning and afternoon temperatures. Temperatures increased gradually from the first week 16.8 ± 0.7 ºC (morning), 18.6 ± 0.7 ºC (afternoon), to the fourth week 20.0 ± 1.2 ºC (morning), 22.9 ± 1.5 ºC (afternoon). There was a slight decrease in temperature in the fifth week 19.9 ± 0.9 ºC (morning), 22.1 ± 0.9 ºC (afternoon), followed by a considerable decrease in the sixth week 17.5 ± 0.6 ºC (morning), 19.9 ± 0.9 ºC (afternoon). Finally, the temperature increased again in the eighth week 20.2 ± 1.0 ºC (morning), 21.3 ± 2.2 ºC (afternoon), and in the ninth week 21.3 ± 0.8 ºC (morning), 23.0 ± 1.1 ºC (afternoon). The weeks that registered highest temperatures are the fourth, fifth, eighth, and ninth weeks, the last one being the one with the highest values.

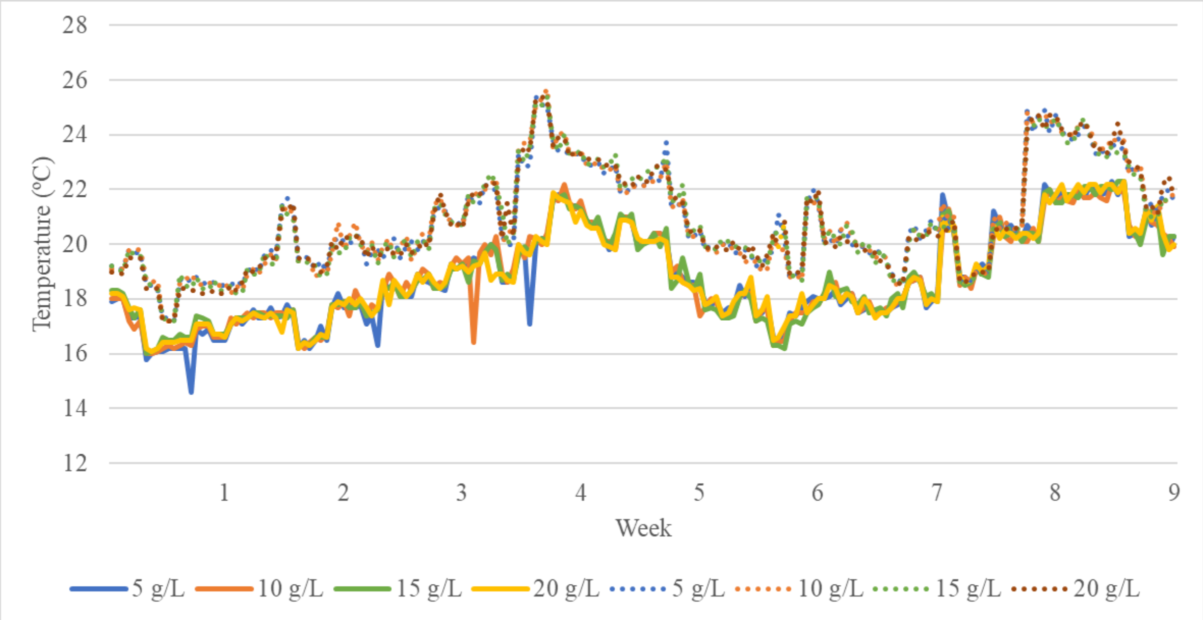

Figure 3.7 Values of temperature in the seaweed cultivation system throughout the weeks (n = 189) Solid-colored lines correspond to morning data and dotted lines correspond to afternoon data.

The variations in oxygen and pH during this experiment were also recorded twice a day (morning and afternoon). For these two parameters, statistical analysis was conducted to determine if there were any differences between densities for each period of the day. The Kruskal-Wallis test showed significant differences between the densities ($p \leq 0.05$) for both parameters and each period of the day. As a post hoc test, the Dunn-Bonferroni test was performed, and the differences ($p \leq 0.05$) are shown in Figures 3.8 and 3.9, respectively, for oxygen and pH. It is relevant to highlight that overall, both in the morning and in the afternoon, oxygen and pH levels are significantly lower at a density of 20 g/L than at 5 g/L and 10 g/L.

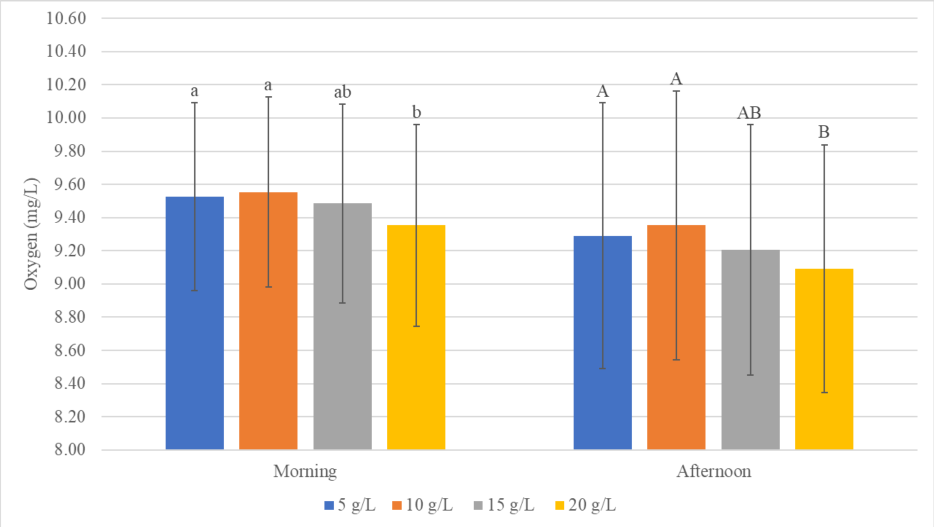

Figure 3.8 Mean of oxygen (mg/L), on morning (n = 186) and afternoon (n = 183) in the seaweed cultivation system. Means sharing a letter do not differ significantly ($p > 0.05$) between densities, with lowercase letters for morning and uppercase letters for afternoon.

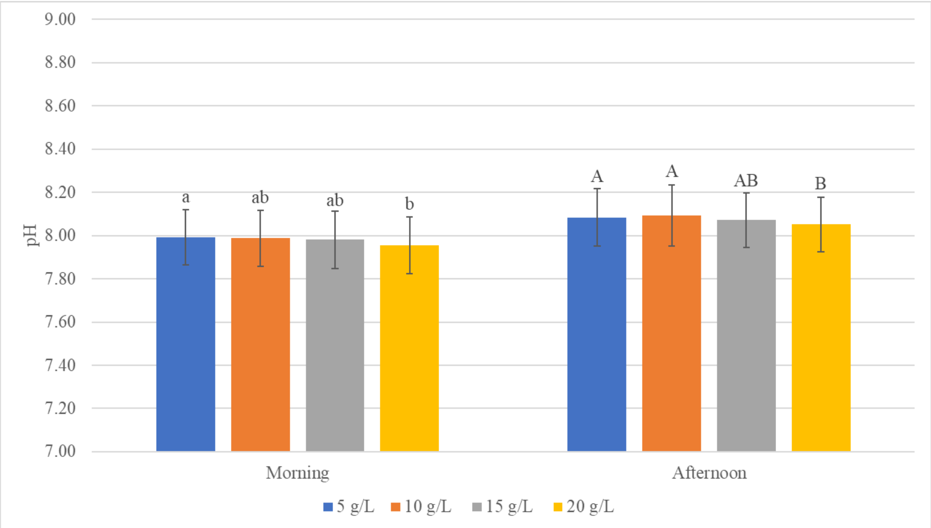

Figure 3.9 Mean of pH, on morning (n = 186) and afternoon (n = 189) in the seaweed cultivation system. Means sharing a letter do not differ significantly ($p > 0.05$) between densities, with lowercase letters for morning and uppercase letters for afternoon.

A Kruskal-Wallis test was performed and showed that there were significant differences on P-PO_4^{3-} over the weeks ($p = 0.030$); however, the Dunn-Bonferroni post-hoc test did not show where these differences were. Nevertheless, the values of P-PO_4^{3-} are shown in Figure 3.10, where it is possible to observe that higher mean P-PO_4^{3-} values (µM) are recorded in the fourth and fifth weeks (1.79 ± 0.18 and 1.68 ± 0.16, respectively) than in the other weeks, and the lowest mean value recorded was in the ninth week (0.51 ± 0.23).

For N-NH_4^+ and N-NO_3^- the statistical tests did not show significant differences over the weeks ($p = 0.795$ and $p = 0.706$, respectively). The average values of N-NH_4^+ and N-NO_3^- (µM) throughout the experiment were 5.24 ± 3.13 and 3.76 ± 2.07, respectively. However, it is also possible to observe from the graph in Figure 3.10 that the average N-NH_4^+ values (µM) were higher in the third and fourth weeks (7.92 ± 5.88 and 7.22 ± 4.86, respectively), and the lowest mean values were recorded in the fifth and second weeks (3.65 ± 2.11 and 3.81 ± 2.20, respectively). Also, in Figure 3.10, it is possible to note that the average N-NO_3^- values (µM) were higher in the seventh, third, and fourth weeks (5.32 ± 2.76; 4.60 ± 1.87; and 4.60 ± 1.67, respectively), and the lowest mean values were recorded in the eight and ninth weeks (2.58 ± 1.12 and 2.59 ± 1.86, respectively).

Furthermore, the Tukey post-hoc test was conducted for N-NO_2^-, and the significant differences ($p \leq 0.05$) are shown in Figure 3.10. Throughout the experiment, the concentration of N-NO_2^- (µM) consistently remained below 0.60 ± 0.04 (being the higher mean value recorded in the fifth week), and the lower mean value recorded was 0.48 ± 0.03 (ninth week).

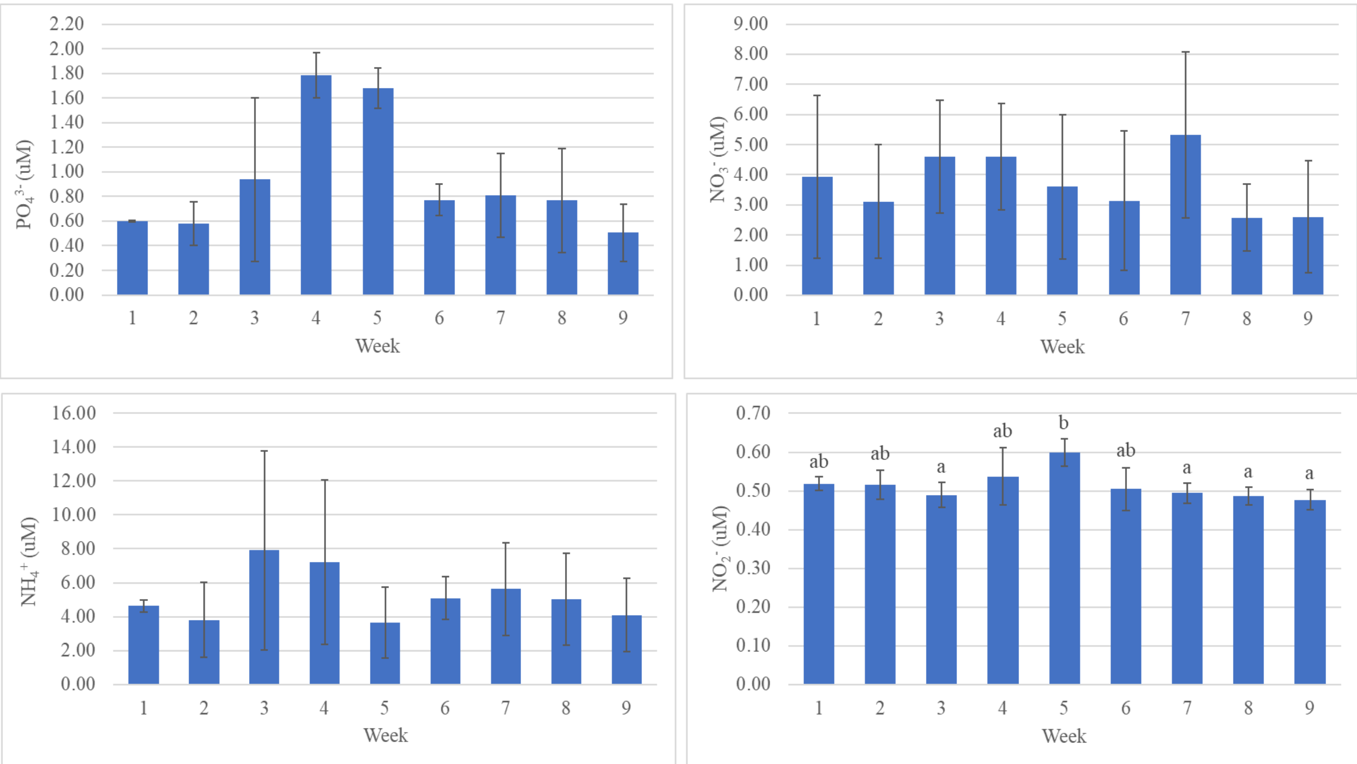

Figure 3.10 Mean of dissolved nutrients P-PO_4^{3-}; N-NH_4^+; N-NO_3^-; and N-NO_2^- (µM) throughout the weeks (n = 3 or 4). Means sharing a letter do not differ significantly ($p > 0.05$).

Additionally, for all nutrients (P-PO_4^{3-}; N-NH_4^+; N-NO_3^-; and N-NO_2^-) to investigate if the nutrient supply differs throughout the day (9h, 12h, 15h, and 18h), represented in Figure 3.11, a one-way ANOVA test was performed and showed that there were no significant differences ($p = 0.909$; $p = 0.524$; $p = 0.149$; $p = 0.624$, respectively). Although there were no significant differences in the quantity of nutrients throughout the day, there was a tendency for the quantity of phosphorus (P-PO_4^{3-}) and ammonium (N-NH_4^+) to be higher at 12h, followed by 9h, 15h, and 18h. Nitrate almost followed the same trend, but 18h had slightly more of this nutrient than 15h. In addition, the daily averages obtained for each nutrient (µM) were 1.16 ± 0.74 (P-PO_4^{3-}); 5.24 ± 3.13 (N-NH_4^+); 3.76 ± 2.07 (N-NO_3^-); and 0.51 ± 0.05 (N-NO_2^-).

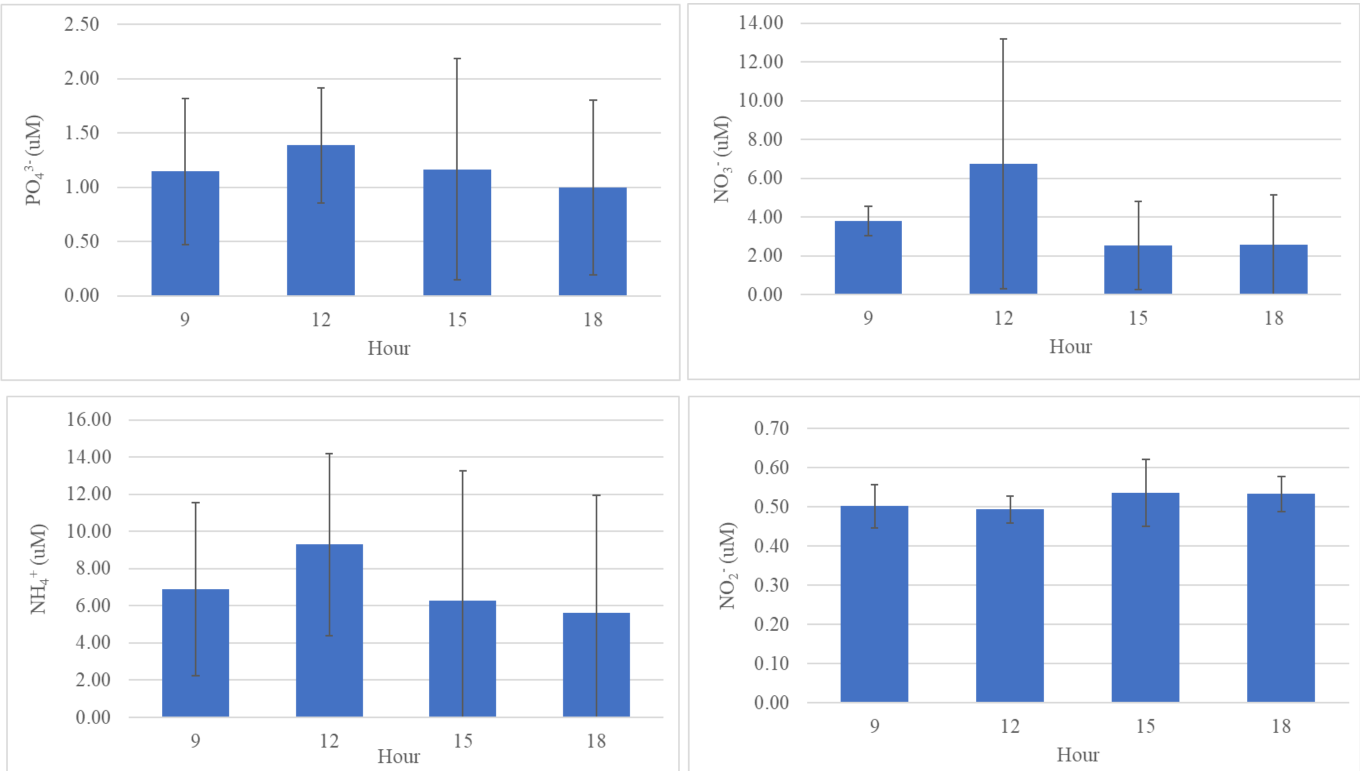

Figure 3.11 Mean values of dissolved nutrients P-PO_4^{3-}; N-NH_4^+; N-NO_3^-; and N-NO_2^- (µM), throughout the day (n = 4 or 5).

3.2.2. *Halopetris scoparia* growth in an IMTA system

The specific growth rate (SGR) of *Halopteris scoparia*, represented in Figure 3.12, shows that *H. scoparia* at a density of 5 g/L has a higher SGR over the weeks than when cultivated at the other tested densities, and there is a tendency that the higher the density, the lower the SGR. The highest mean values of SGR (% FW d^{-1}) registered were

10.65 ± 1.97 and 10.07 ± 2.36 at the seventh and ninth weeks, respectively, both at the density of 5 g/L.

To determine if the SGR differed according to the different stocking densities, a one-way ANOVA was performed for the first, second, third, fourth, and sixth weeks, and a Kruskal-Wallis was performed for the fifth, seventh, eighth, and ninth weeks. In all weeks, the tests showed significant differences (represented in Figure 3.12) in the SGR between densities ($p \leq 0.05$). It should be noted that in all weeks, the 5 g/L density has a significantly higher SGR than the 20 g/L density ($p \leq 0.01$ from the first to the sixth weeks and in the eighth week; and $p = 0.01$ for fifth week and $p = 0.007$ for the seventh and ninth week). Furthermore, the 10 g/L density also has a significantly higher SGR than the 20 g/L density between the first and fourth weeks (with $p = 0.003$; $p = 0.005$; $p = 0.003$ and $p = 0.001$, respectively), sixth ($p = 0.003$) and eighth ($p = 0.001$) weeks. In addition, 5 g/L density has a significantly higher SGR than the 15 g/L density in the second, third, fourth, sixth, and eight weeks (with all $p < 0.001$) and 10 g/L density also has a significantly higher SGR than the 15 g/L density in the second ($p = 0.015$), third ($p = 0.021$), fourth ($p = 0.005$), sixth ($p = 0.042$), and eight ($p < 0.001$) weeks.

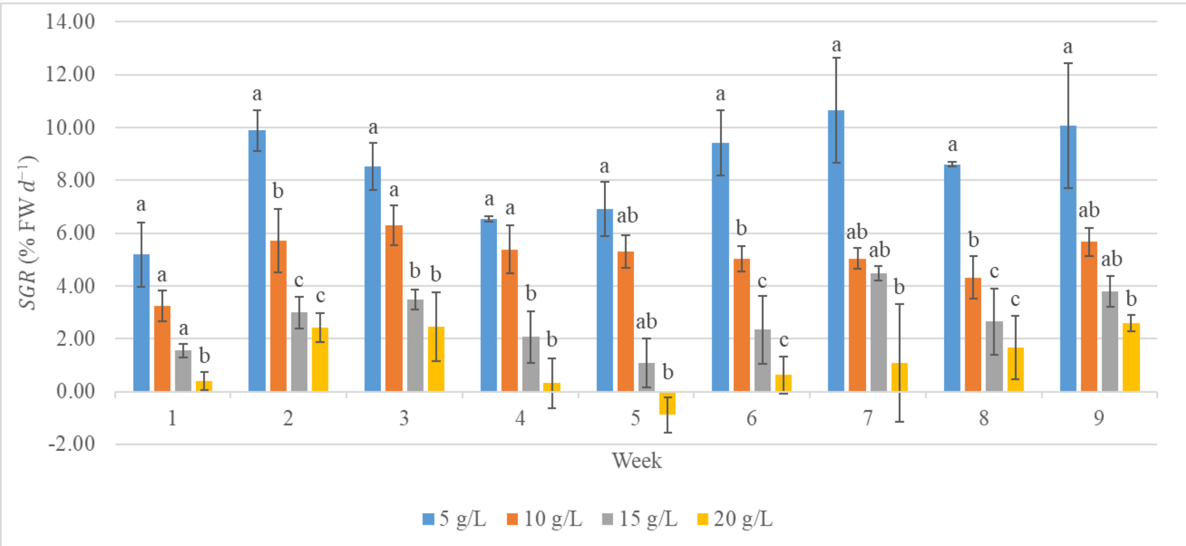

Figure 3.12 Mean of specific growth rate throughout the weeks of *H. scoparia* (n = 3) in IMTA. The bars represent the mean values of SGR for each density (5, 10, 15 and 20 g/L). Within each week, means sharing a letter do not differ significantly between densities ($p > 0.05$).

For each density, to determine if the SGR differed throughout the weeks (time), a one-way ANOVA was performed for 15 g/L, and a Kruskal-Wallis was performed for 5 g/L, 10 g/L, and 20 g/L. At the density of 5 g/L, the Kruskal-Wallis test shows significant differences; however, the Dunn-Bonferroni post-hoc test does not show where the differences were ($p > 0.05$). For the 10 g/L density, the test does not show significant differences in the SGR between weeks ($p > 0.05$). Only for the densities of 15 g/L and 20 g/L did the tests show where the differences were between the weeks. In order to better visualize these differences, they are shown in Figure 3.13. For the density of 15 g/L, the significantly higher SGR (4.49 ± 0.28% FW d^{-1}) was achieved at the seventh week, and the significantly lowest SGR (1.09 ± 0.92% FW d^{-1}) was achieved at the fifth week. Finally, for the density of 20 g/L, the Dunn-Bonferroni post-hoc test showed that the ninth week had significantly higher SGR (2.59 ± 0.32% FW d^{-1}) than the fifth week ($p = 0.051$), being this one a negative value.

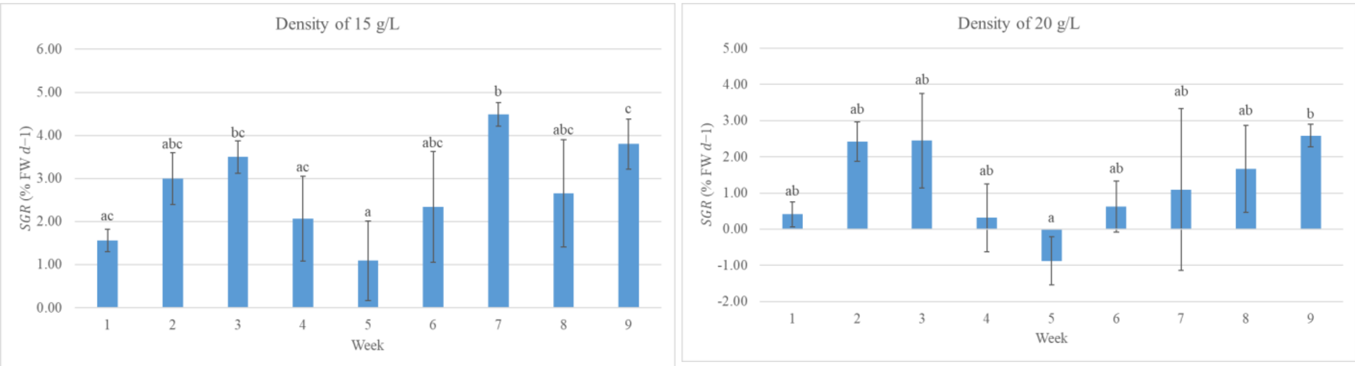

Figure 3.13 Mean of specific growth rate throughout the weeks of *H. scoparia* (n = 3) at the density of 15 g/L and 20 g/L in IMTA. Means sharing a letter do not differ significantly ($p > 0.05$) between the nine weeks.

Finally, figure 3.14 represents the mean yield values for *H. scoparia*, showing that the density of 10 g/L has the highest yield until the fifth week (inclusive), while the density of 5 g/L has the highest yield from the sixth to the ninth week (inclusive). The highest mean values of yield (g FW L wk^{-1}) registered were 1132.77 ± 161.10 (10 g/L, third week) and 1096.07 ± 161.10 (5 g/L, seventh week).

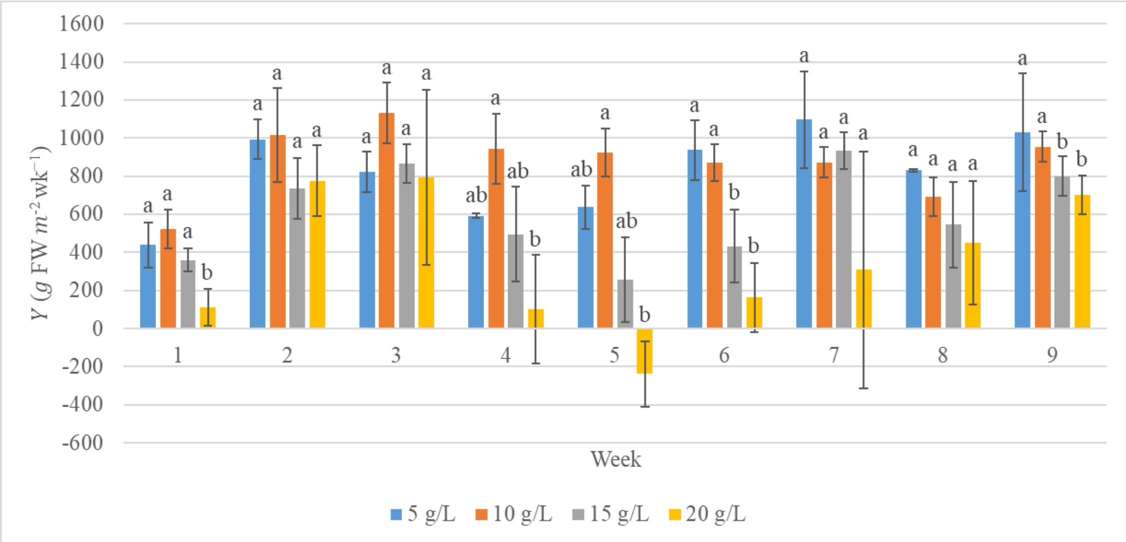

Figure 3.14 Mean of yield throughout the weeks of *H. scoparia* (n = 3) in IMTA. The bars represent the mean values of yield for each density (5, 10, 15 and 20 g/L). Within each week, means sharing a letter do not differ significantly between densities ($p > 0.05$).

To determine if the yield differed significantly according to the different stocking densities, a one-way ANOVA was performed for the first, second, fourth, and sixth, eight, and ninth weeks, and a Kruskal-Wallis was performed for the third, fifth, and seventh weeks. In all weeks in which significant differences were found between densities (weeks 1, 4, 5, and 6), the 10 g/L yield was significantly higher than the 20 g/L yield ($p \leq 0.05$). Although in the remaining weeks (weeks 2, 3, 7, 8, and 9) no significant differences were found between densities ($p > 0.05$); however, it is possible to observe that the 10 g/L yield is still higher than the 20 g/L yield.

For each density, to determine if there were statistical differences in the yield throughout the weeks, a one-way ANOVA was performed for the densities of 10 g/L and 15 g/L, and a Kruskal-Wallis was performed for the densities of 5 g/L and 20 g/L. Regarding the statistical analysis throughout the weeks, the significant differences are represented in Figure 3.15. However, there were no significant differences that were consistent across all densities**.**

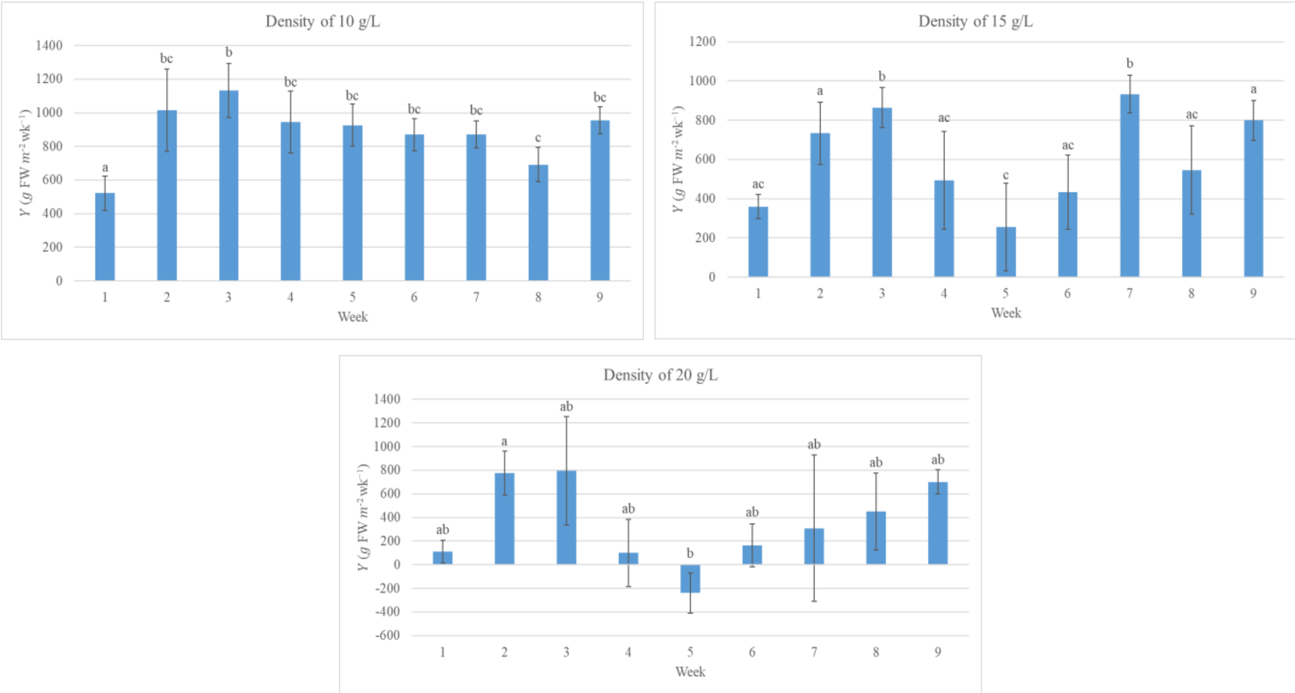

Figure 3.15 Mean of yield throughout the weeks of *H. scoparia* (n = 3) at the densities of 10, 15 and 20 g/L in IMTA. Means sharing a letter do not differ significantly ($p > 0.05$) between the nine weeks.

3.2.3. C:N content

It was analyzed whether the content of nitrogen (%N), carbon (%C) and C:N ratio was significantly different between the different cultivation densities (5, 10, 15, and 20 g/L) and over time (weeks 0, 4, and 9). The values from the first sampling correspond to the four densities, since the initial biomass was used to supply all the tanks.

The nitrogen (% N DW) in the initial biomass of *H. scoparia* was 2.2 ± 0.06%. The highest mean values of N content were 3.9 ± 0.10% (5 g/L) and 3.9 ± 0.06% (10 g/L) both on the final week. A Kruskal-Wallis test was performed and only showed differences between densities in the fourth week, although the Dunn-Bonferroni post-hoc test failed to identify where the differences were. However, it can be noted that the highest nitrogen content was at a density of 10 g/L, followed by 5 g/L, in both weeks.

To determine if the N content differed throughout the weeks, a Kruskal-Wallis test was also performed for each density (5, 10, 15, and 20 g/L) and showed that there were significant differences between weeks for all densities ($p = 0.026$; $p = 0.025$; $p = 0.025$; $p = 0.046$, respectively). Consequently, a Dunn-Bonferroni post-hoc test was performed and showed that the N content from the beginning (initial biomass) was significantly lower than in the end (ninth week) for all densities ($p = 0.011$, $p = 0.010$, $p = 0.010$, and $p = 0.023$, respectively), as shown in Figure 3.16.

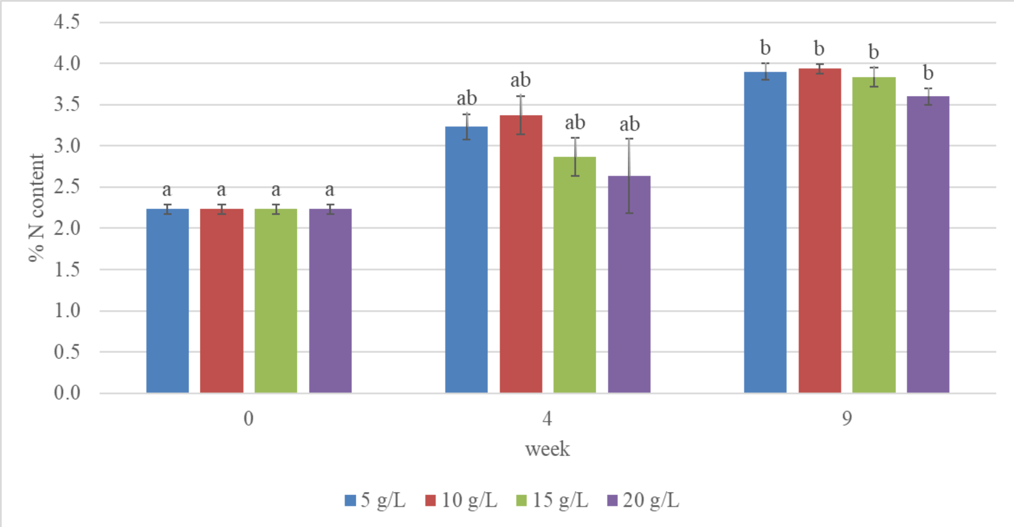

Figure 3.16 Mean values of nitrogen content, at the beginning, middle and end of the cultivation, of *H. scoparia* (n = 3) in IMTA. The bars represent the mean values of nitrogen content for each density (5, 10, 15 and 20 g/L). Within each density, means sharing a letter do not differ significantly between sampling times ($p > 0.05$).

The carbon content (% C DW) is shown in Figure 3.17. The carbon content of the initial biomass of *H. scoparia* recorded was 28.2 ± 1.27%. The highest mean value was 30.9 ± 1.21% at a density of 15 g/L in the ninth week. According to the Kruskal-Wallis test, there are no significant differences in the algae carbon content at the different densities in the fourth and ninth weeks ($p = 0.219$; $p = 0.096$, respectively).

To determine if the carbon content differed throughout the weeks, for the densities of 5 and 10 g/L, a Kruskal-Wallis test, and for the densities of 15 g/L and 20 g/L, a one-way ANOVA test was performed. These tests showed that there are significant differences ($p \leq 0.05$) throughout the weeks. Subsequently, the Dunn-Bonferroni and Tukey post-hoc tests, respectively, were performed, and the significant differences are shown in Figure 3.17. Overall, the %C content in algae is significantly higher in the ninth week than in the fourth week (with $p = 0.037$ for 5 g/L, $p= 0.011$ for 10 g/L, and $p < 0.001$ for 15 and 20 g/L).

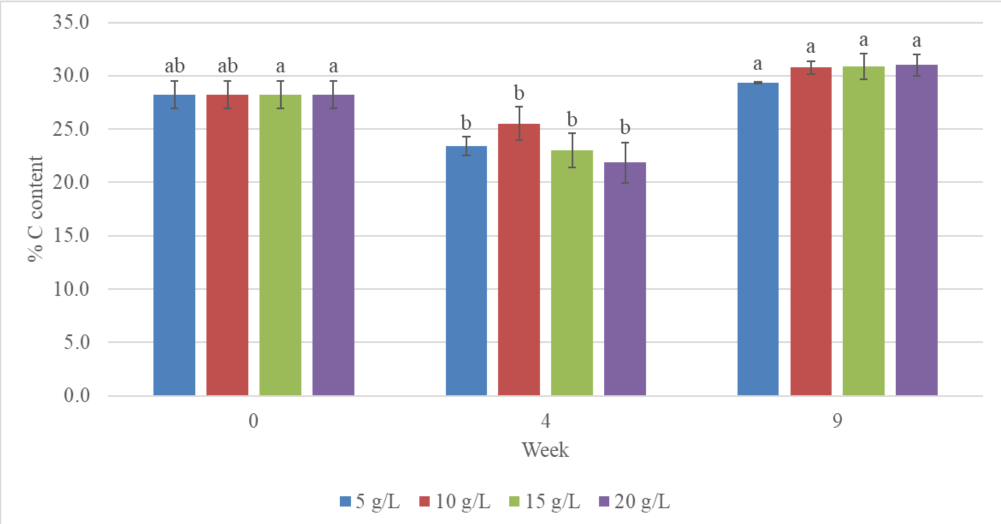

Figure 3.17 Mean values of carboncontent, at the beginning, middle and end of the cultivation, of *H. scoparia* (n = 3) in IMTA. The bars represent the mean values of carbon content for each density (5, 10, 15 and 20 g/L). Within each density, means sharing a letter do not differ significantly between sampling times ($p > 0.05$).

In order to analyze if the C:N ratio differed according to the stocking densities, a Kruskal-Wallis and a one-way ANOVA test were performed for the middle and final weeks, respectively. Both showed that there are significant differences between densities ($p = 0.023$ and $p < 0.001$, respectively). The respective post-hoc Dunn-Bonferroni and Tukey tests were performed and showed where the differences are, as shown in Figure 3.18. Furthermore, it is relevant to mention that for both samplings, the density of 5 g/L showed a significantly lower content of C:N than the densities of 15 ($p = 0.051$ and $p = 0.009$, for fourth and ninth weeks, respectively) and 20 g/L ($p = 0.019$ and $p = 0.001$, for fourth and ninth weeks, respectively).

To analyze if there are differences over time, a Kruskal-Wallis test was performed for 5 g/L and 10 g/L, and a one-way ANOVA test was performed for 15 g/L and 20 g/L. For all densities, the tests performed show that for each density, the C:N varies significantly throughout the experience. The correspondent post-hoc tests were performed and show that for 5, 15 and 20 g/L densities, the C:N is significantly higher on the first sampling (initial biomass) than on the fourth and ninth weeks (all with $p < 0.001$), and 10 g/L density is only significantly higher on the first week comparing to the fourth week ($p = 0.011$). However, the value of the content of C:N on the ninth week is still lower than on the first sampling, as it is possible to observe in the figure 3.18.

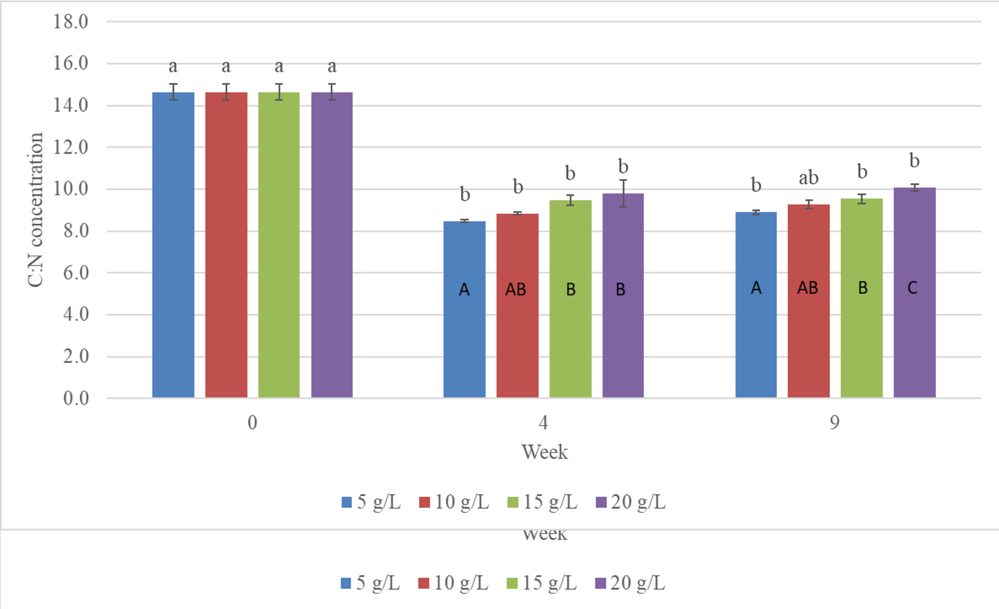

Figure 3.18 Mean values of C:N, at the beginning, middle and end of the cultivation, of *H. scoparia* (n = 3) in IMTA. The bars represent the mean values of carbon to nitrogen ratio content for each density (5, 10, 15 and 20 g/L). Within each week, means sharing an uppercase letter do not differ significantly between densities ($p > 0.05$). Within each density, means sharing a lowercase letter do not differ significantly throughout cultivation ($p > 0.05$).

3.2.4. N-yield

From the N content and the biomass yield of the algae, the nitrogen removal capacity of *H. scoparia* was calculated and referred to as the N-yield. A two-way ANOVA was performed to test if the different densities, time of cultivation, and the interaction of the two factors have an impact on N-yield. The test shows that there are significant differences between the densities of cultivation and throughout time (both with $p < 0.001$) and that there was no significant interaction between the two factors ($p = 0.192$). Following, a Tukey post-hoc test was performed and shows that the N-yield was significantly higher in the ninth week (final of the experiment) than in the fourth week (middle of the experiment), represented in Figure 3.19 ($p < 0.001$). The Tukey post-hoc test also shows that the N-yield is significantly higher at a density of 5 g/L than 20 g/L ($p = 0.006$) and is significantly higher at a density of 10 g/L than at a density of 15 and 20 g/L ($p = 0.033$ and $p < 0.001$, respectively), represented in figure 3.20.

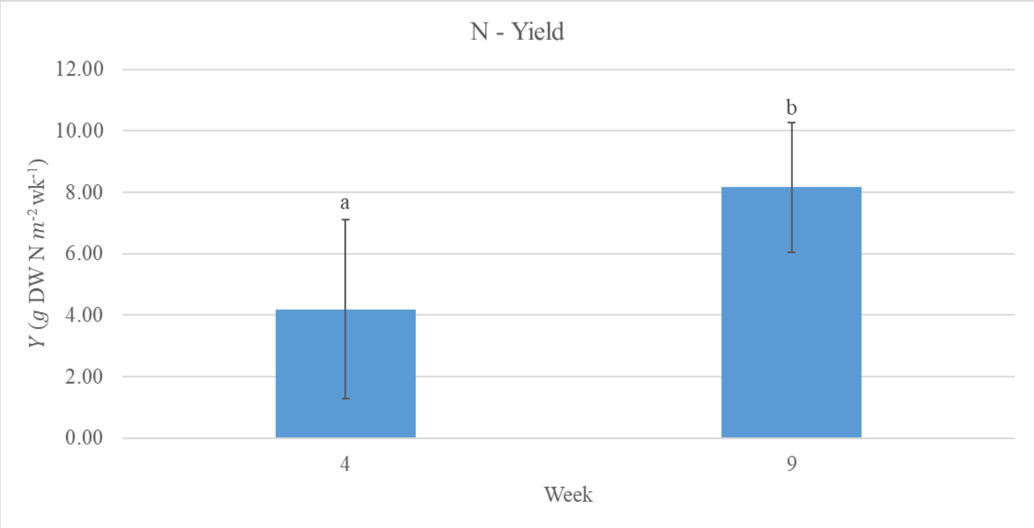

Figure 3.19 Mean values of N-yield at the middle and end of the cultivation of *H. scoparia* (n = 12) in IMTA. Means sharing a letter do not differ significantly ($p > 0.05$).

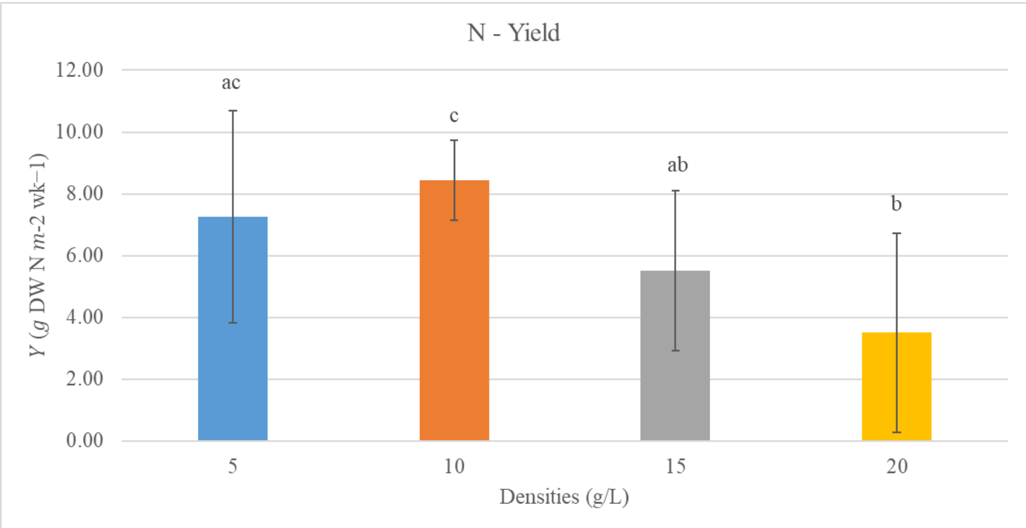

Figure 3.20 Mean values of N-yield of *H. scoparia* (n = 6) in IMTA. The bars represent the mean values of carbon content for each density (5, 10, 15 and 20 g/L). Means sharing a letter do not differ significantly ($p > 0.05$).

4. Discussion

In the first experiment, the potential of cultivation of *C. tomentosum* and *H. scoparia* was evaluated using two different methods: tumble culture and attachment to the substrate. For both cultivation methods and both species, the pH and salinity values, overall, increased over the weeks. One possible reason for the increase in pH is that the removal of carbon dioxide by macroalgae increases the pH (Boyd, 2015). Some studies show that higher pH values (>8.5, >9, or >9.5, depending on the species) affect the capacity to use bicarbonate (HCO_3^-) as a carbon source and affect or completely inhibit photosynthesis and growth (Blinks, 1963; Lise & Juel, 2007; Zou & Gao, 2009; Zou *et al.*, 2004). The increase in salinity may be because of technical issues, as the inability to finely control the stirring of the water caused water accumulation on the parafilm, leading to its displacement. This caused water loss and evaporation, which increased the salinity of the medium. So, it was concluded that the use of parafilm to cover the beakers is not the most efficient method. Therefore, it is suggested to use glass bottles covered with a cork that only has a hole for the air tube or cotton stoppers covered with gauze.

Furthermore, the increase in salinity may have affected the growth of *C. tomentosum* since, according to Yang et al. (1997), the optimum growth for *C. fragile* ssp. *tomentosoides* occurred at 23.8% (at 20 ºC). Additionally, Hanisak (1979a) reported optimum thalli growth of *C. fragile* ssp. *tomentosoides* at 24 and 30 PSU salinity at temperatures of 18 ºC (the same temperature used in this study). Hanisak (1979a) study also obtained that the thalli grew at 24 ºC with salinity between 12 PSU and 42 PSU and died at a salinity of 6 PSU and 48 PSU (minimum and maximum salinity tested, respectively) and that the survival and growth of thalli of *C. fragile* ssp. *tomentosoides* at a given salinity were dependent on temperature. Furthermore, according to Yang et al. (1997), different *Codium* species preferred different salinities to promote optimal growth, and *C. tomentosum* achieved its best growth at a salinity of 30.6 PSU. According to Silva (2011), *H. scoparia* grows at a wide range of salinities (between 20 – 46 PSU), but the growth rate of *H. scoparia* is favored at a salinity of 20 PSU.

Regarding the contamination by epiphytic algae on tumble cultivation, for both species and densities, a notable increase in contamination was observed throughout the cultivation weeks. Similarly, the SGR and yield also increased throughout the cultivation weeks. These rising values may be related to the presence of epiphytic algae. Patarra et al. (2017) obtained the highest growth rates at 150 μmol photons m^{-2} s^{-1}; however, at this light intensity, they obtained a greater abundance of epiphytes, suggesting that the increase in growth rates may be more due to the incidence of epiphytes than to the species under study.

On the other hand, the length of *C. tomentosum* increased throughout the cultivation for both densities; however, *H. scoparia* length was significantly higher in the early weeks than in the subsequent weeks for both densities. This indicates that, throughout the experiment, *C. tomentosum* continues to grow in length regardless of the epiphytic algae, but *H. scoparia* did not grow in length, suggesting that this species is more susceptible to contamination by epiphytic algae. The results of *H. scoparia* emphasize that the SGR and yield values may be influenced by the presence of epiphytes. Nevertheless, in the final stage of cultivation, both species do not have the color and vitality that they had at the start. Regarding the light intensity used in this study, which ranged between 70 – 100 µmol photons m^{-2} s^{-1}, it may have influenced the high existence of epiphytes. According to Patarra et al. (2017), the best overall growth performance was obtained at 70 µmol photons due to the influence of light intensity on epiphyte abundance.

Furthermore, at both cultivation methods and species, *C. tomentosum* and *H. scoparia* showed visually more contamination at the lower densities. These results may be because the lower densities are more exposed to light and have more nutrients available. The abundance and biodiversity of epiphytes depends on the morphology of the cultivated species and on abiotic factors such as the season, water movement, and nutrient availability (Kersen *et al*., 2011; Sahu *et al*., 2020). In some studies, higher irradiances and amounts of nutrients were coincident with higher amounts of epiphytes (Kersen *et al*., 2011; Patarra *et al*., 2017).

Regarding the substrate cultivation of *H. scoparia*, it is worth noting that some of the observed contamination by other algae might have been *C. tomentosum*, given its similar appearance (circular green dots). In this experiment, it can be inferred that it is necessary to improve this cultivation method so that there is no contamination by other algae and that it could have been a favorable approach to work with each species at separate times, or just one, to reduce the contamination of one by the other. In addition, it is crucial to note that although visual observations of epiphytes provide useful insights, quantitative analyses are required to complement these observations for more robust conclusions.

In substrate cultivation of *C. tomentosum*, it is not clear that there was attachment; however, at the second week, circular green dots appeared, which can be *C. tomentosum*. Additionally, it is important to confirm that it is really *C. tomentosum* that is growing attached to the twine, so a longer cultivation should be carried out. In the study by Hwang et al. (2005), the maximum growth of zygote attachment (sexual reproduction) of *C. fragile* (Suringar) was obtained after 13 days, and the maximum regeneration of isolated medullary filament was obtained after 15 days of culture, which aligns with the timelines observed in this study. Over the weeks, more green dots appeared and became more evident. However, to promote the attachment of *C. tomentosum* to the twines, the spools could have been left in the solution of triturated macroalgae for longer, as was done in the study by Nanba et al. (2005), in which it was left for a week. Although there is no statistical analysis of the pictures, visual observation indicates that the higher density (65 g/L) has a higher number of green dots, so it can be considered the best cultivation density. Additionally, it is important to note that the methodology adopted for analyzing the green dots, which involves sampling pieces of twine, may limit the results. Once, it is impossible to examine the entire twine due to its length. Regarding the spore attachment of *H. scoparia* to the twine, it remains unclear whether this occurred.

Finally, it is crucial to consider that the weekly sampling may have affected the species attachment to the twine, inhibiting their growth and/or causing detachment. The handling during the medium change could have led to the species becoming detached and/or lost. To enhance the substrate cultivation method, some improvements could be made: reducing the frequency of sampling (allowing more stable attachment of the macroalgae species to the twine) and using smaller pieces of twine that can be fully analyzed on both sides. Additionally, future studies should investigate how water movement (e.g., from aeration) affects the attachment and growth of the macroalgae species, similar to how Nanba et al. (2005) investigated the effect of water flow on the formation and growth of spongy and filamentous thalli of *Codium fragile*.

Concerning the tumble cultivation of *C. tomentosum*, at the beginning (initial length), although the pieces of seaweed were randomly chosen, the 8 g/L density had significantly smaller pieces of seaweed than the 4 g/L density. This could be due to random selection or error associated with the measurement of macroalgae from photographs. Furthermore, in the first week both densities showed similar lengths; this indicates that the 8 g/L density grew, and the density of 4 g/L did not. Comparing the results with the existing bibliography, it was found that an average SGR (% FW d^{-1}) of 4 g/L (7.35 ± 3.96) and 8 g/L (4.60 ± 1.75) exceeded the 4% daily growth rate also in lab essays (Rego *et al*., 2014). Additionally, the mean length growth (mm d^{-1}) of both densities (0.086 ± 0.081 and 0.106 ± 0.036 respectively, for 4 g/L and 8 g/L) was lower than that obtained by Hwang et al. (2008), which was 0.113 ± 0.003 mm d^{-1}.

Regarding the *H. scoparia* tumble cultivation, the SGR and yield were higher at a density of 2 g/L than 4 g/L. However, 4 g/L had visually less contamination and achieved a significantly higher length than 2 g/L, suggesting it might be a more favorable cultivation density. In this cultivation, the mean SGR (% FW d^{-1}) values were 10.18 ± 4.32 (2 g/L) and 5.58 ± 2.59 (4 g/L), both at 18 ºC, light intensity between 70–100 µmol photons m^{-2} s^{-1}, and a photoperiod of 12:12. Silva (2011) obtained the best mean growth rate of 6.8% FW day^{-1}, similar to that obtained at 4 g/L density, but at a lower cultivation density (0.1 g/L) and temperature (15 ºC) and at a higher light intensity of 150 µmol photons m^{-2} s^{-1}. Patarra et al. (2017) obtained growth rates of around 9% FW day^{-1}; 18 and 24 °C; and at 150 µmol photons m^{-2} s^{-1}, similar to those obtained at 2 g/L results but at a higher light intensity. However, Patarra et al. (2017) considered that the best overall growth performance (between RGR and productivity) was obtained when cultivated at a light intensity of 70 µmol photons m^{-2} s^{-1} and 18 ºC, reaching 4.6% day^{-1} (RGR), like that obtained at 4 g/L density. In an IMTA system, Patarra et al. (2016) obtained optimal growth at a lower density of 2 g/L, achieving a RGR of 5.52 ± 1.84% day^{-1}, which was lower than the results obtained at both densities. However, these comparisons of results of SGR with RGR are subjective since the cultivation method is different and the growth calculation formulas are not the same.

The second experiment consisted of cultivating *Halopteris scoparia* in an IMTA system to evaluate how different culture densities (5, 10, 15, and 20 g/L) affect its growth, yield, and bioremediation potential. Overall, the growth of *H. scoparia* was affected by the cultivation density.

Some studies have revealed that *H. scoparia* has the ability to grow in diverse temperature ranges (Novaczek *et al*., 1989; Silva, 2011), and accordingly, in this study, *H. scoparia* grew between temperatures of 16.6 ºC (morning) and 23.1 ºC (afternoon). Moreover, a higher light intensity also seems to favor *H. scoparia* growth. During the experiment, there was a transition from spring to summer, which consequently led to an increase in photoperiod and light intensity. Accordingly, Patarra et al. (2017) obtained higher growth rates at 18 and 24 ºC and at 150 µmol photons m^{-2} s^{-1} than Silva (2011) obtained at 15 ºC and 50 µmol photons m^{-2} s^{-1}. Furthermore, when the nutrient concentrations in the water were higher, particularly $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$, the growth of *H. scoparia* seemed to be favored. Silva et al. (2011) study shows that *H. scoparia* preferentially removed $N\text{-}NH_4^+$ over $N\text{-}NO_3^-$. Although the objectives of this study were not to determine the ideal growth temperatures or nitrogen requirements of *H. scoparia*, it is noted that further research is required to determine the ideal conditions for growing *H. scoparia*.

As can be seen in Annex 6, and according to the sampling annotations, the lower the density, the higher the amount of epiphytes. Over the experiment, the number of epiphytic algae increased and the appearance of *H. scoparia* deteriorated. The lowest density (5 g/L) was the first to show a higher amount of epiphytes and signs of deteriorating health in *H. scoparia*, followed by the 10 g/L density, which exhibited similar issues. The 15 g/L density then experienced more epiphytes and noticeable algae fragmentation. The highest density (20 g/L) was the last to display signs of epiphytes and declining health. Patarra et al. (2017) obtained a higher abundance of epiphytes at the highest light intensity. These results are consistent with those obtained in this experiment, since the lower densities are more exposed to light. Moreover, the signs of deterioration in the health of the macroalgae starting to be more evident from lower to higher densities may also be related to the increase in photoperiod and light intensity throughout cultivation.

According to the Kim et al. (2013) study, growth rate was inversely related to stocking density. This inverse relationship was also observed in this study, where lower stocking densities (5 g/L and 10 g/L) resulted in higher SGR and yields of *H. scoparia*. Thus, the higher SGR, yield, and N-yield values recorded by the lower densities may have been favored by the more available light and greater nutrient availability**,** leading to a higher rate of photosynthesis and consequently better growth results (Abreu *et al*., 2011; Demetropoulos and Langdon, 2004; Pereira *et al*., 2006). Although Patarra et al. (2017) reported significantly higher RGR and productivity of *H. scoparia* at higher light intensity, they also observed a greater abundance of epiphytes, suggesting that the higher growth rates may be attributed to the higher incidence of epiphytes than to the growth of the target species itself. Which could also have happened in this experiment. Additionally, higher densities led to lower growth, which may be related to the fact that higher densities have more intense competition for resources. Accordingly, it was noted that the higher densities (15 g/L and 20 g/L) must have had N-limitation, and it is known that nitrogen is an essential macronutrient and a limiting factor for the growth of macroalgae (Roleda & Hurd, 2019; Sheppard *et al.*, 2023). Moreover, higher densities could have lower photosynthetic rates. Indicating that even with the aeration placed at the bottom of the tanks, which favors the distribution of macroalgae and consequently allows them to access light (Abreu *et al.*, 2011), it was not enough. Consequently, these two factors (possible N-limitation and lower photosynthetic rates) lead to poor growth and productivity of the macroalgae at the higher densities (15 g/L and 20 g/L) (Abreu *et al.*, 2011; Kang *et al.*, 2021; Kim *et al.*, 2013; Roleda & Hurd, 2019).

According to two studies, which cultivated *H. scoparia* under controlled conditions, they obtained growth rates (% FW day^{-1}) of 6.8% at 0.1 g/L (Silva, 2011) and 9.45 ± 1.61% at 2 g/L (Patarra *et al.*, 2017). According to Patarra et al. (2016), who cultivated *H. scoparia* in an IMTA system, the growth rate (% day^{-1}) obtained was 5.52 ± 1.84 at 2 g/L. When comparing the SGR (% FW d^{-1}) results from this study. It was obtained that the average SGR value of 8.42 ± 2.07 (5 g/L) was higher than that obtained by Patarra et al. (2016), another study in an IMTA system, and that Silva (2011) under controlled conditions. However, was not higher than those obtained by Patarra et al. (2017), also in controlled conditions. In contrast, the average SGR (% FW d^{-1}) value of 5.11 ± 1.05 (10 g/L) was almost identical to those obtained by Patarra et al. (2016) and lower than Silva (2011) and Patarra et al. (2017).

Analyzing the nitrogen (%N) content in the biomass of *H. scoparia*, it was found that in all densities, the %N content was significantly lower in the initial sample than in the ninth week. This initial sample corresponds to the algae sampled from the coast. So, this significant difference is probably due to the fact that nutrient concentrations are lower in the sea. When the C:N value is higher, it suggests nitrogen limitation (Ahn *et al*., 1998; Gómez Pinchetti *et al*., 1998; Hanisak, 1990; Vergara *et al*., 1993). Accordingly, the C:N ratio was significantly higher initially (week 0), indicating N-limitation at week 0 (Ahn *et al.*, 1998; Gómez Pinchetti *et al.*, 1998; Hanisak, 1990; Vergara *et al.*, 1993). Moreover, de C:N values were significantly higher at the higher densities (15 and 20 g/L) than in the lower densities (5 and 10 g/L), which suggests N-limitation at the higher densities. Furthermore, the N-yield was significantly higher at the lower densities than the higher densities.

Kang et al. (2021) investigated the bioremediation potential of five macroalgae species (of the three Phyla) in an IMTA system. At the end of the experiment, the N content (%) in the biomass ranged from 1.38% in *Ecklonia stolonifera* (Phaeophyceae) to 2.10% in *Ulva pertusa* (Chlorophyta), and the highest value obtained was 2.43 ± 0.16% in *Gracilariopsis chorda* (Rhodophyta) (Kang *et al.*, 2021). In the study conducted by Yu et al. (2016), *Sargassum hemiphyllum* (Phaeophyceae) was cultivated on an oyster farm, and the total results of N content in the biomass were similar to the previous ones, being in the range of 1,79 – 2,52%. In contrast, Chung et al. (2002) cultivated several species of macroalgae (of the three Phyla) with nutrient-rich effluents and obtained higher nitrogen contents. The highest nitrogen values in macroalgae biomass were obtained in *Porphyra* (Rhodophyta) up to 5.7% (Chung *et al.*, 2002). The values obtained by the other macroalgae were 4.7% in *Sargassum* spp. (Phaeophyceae); 4.6% in *Enteromorpha* spp. (Chlorophyta); and 4.2% in *Undaria* spp. (Phaeophyceae) (Chung *et al.*, 2002). Comparing the results of this study with the literature, it was found that our values of %N content (3.4 ± 0.23 (10 g/L); 3.2 ± 0.15 (5 g/L); 2.9 ± 0.23 (15 g/L); and 2.6 ± 0.45 (20 g/L)) in the *H. scoparia* biomass are in agreement with those obtained in other studies, and also that all densities have higher nitrogen (N) content in the biomass than the two studies mentioned.

The carbon content in the biomass of macroalgae differs between species (Yang *et al.*, 2015). In this study, the carbon content (%C) in the tissue of *H. scoparia* does not show significant differences between the densities, so the stocking densities seem to not have a significant impact on carbon removal. The average value of the carbon content (%C) was 23.44 ± 1.52 (fourth week) and 30.50 ± 0.78 (ninth week). The values obtained in this experiment are within the range of values obtained in other studies with brown macroalgae, such as: 25–31% in *Laminaria*; 32-34% in *Ecklonia*; and 33-37% in *Sargassum* (Muraoka *et al.*, 2004); and also 26.55 ± 1.40% in *Laminaria*; 27.39% in *Undaria*; and 28.39 ± 2.14% in *Sargassum fusiforme* (Zheng *et al.*, 2019); and 27.98 ± 1.33% in *Sargassum fusiforme* (Tian *et al.*, 2023). On the other hand, at all densities, the carbon absorbed in the ninth week was significantly higher than that absorbed in the fourth week. This may be due to the transition from spring to summer and consequently the change in environmental conditions, as mentioned above, which may have contributed to the uptake of carbon by the macroalgae (Abreu *et al.*, 2011).

Overall, according to all the data, and at these conditions, 10 g/L seems to be the best density since it has the highest SGR and yield results and a significantly higher N-yield.

5. Conclusion

In the first experiment, at both cultivation methods, the increase in salinity and pH could have affected the growth of both species. Additionally, contamination by epiphytic algae was a recurring problem, especially at lower densities. Regarding the cultivation on substrate, circular green dots were visually observed in the *C. tomentosum* twine, which could be *C. tomentosum*; however, longer cultivation would be necessary to confirm that. In contrast, the cultivation of *H. scoparia* on substrate did not result in the successful attachment or development of new individuals from spores. This study has demonstrated the potential for cultivating *C. tomentosum* and *H. scoparia* but also highlighted several challenges. Future research should focus on: improving methods to reduce contamination by epiphytic algae; testing different methodologies for covering flasks to prevent evaporation; determining optimal pH, salinity, and stocking densities to improve optimal growth; investigating the influence of water movement and sampling (changing the growing medium) on the attachment to the substrate and the influence of the time left in the solution of triturated macroalgae on the attachment to the substrate; and finally developing quantitative analyses to support visual observations and provide more robust conclusions.

Concerning the cultivation in an IMTA system, it was concluded that the lowest densities (5 g/L and 10 g/L) had the highest values of SGR, yield, and N-yield; however, they also had a higher number of epiphytes than the highest densities (15 g/L and 20 g/L). Moreover, the higher densities possible had N-limitation, which can affect their growth. There is a notable disparity between the abundance of studies exploring the potential applications of *Halopteris scoparia* and those focusing on its cultivation methods. Although this macroalgae is promising for various uses, the lack of emphasis on cultivation is concerning, as it implies a continued dependence on wild harvesting. It is therefore imperative to establish efficient cultivation techniques for this species. Given the susceptibility of *H. scoparia* to contamination by other algae, future studies should focus on improving methods for removing and controlling epiphytes (e.g., shaded vs. unshaded cultivations to determine if it is possible to reduce the number of epiphytes). Additionally, further research is needed on optimal cultivation densities, including the higher densities of 15 g/L and 20 g/L, while ensuring adequate nutrient availability. Also, investigate how much nitrogen is needed to maximize the growth of *H. scoparia* and determine its preferred nitrogen source.

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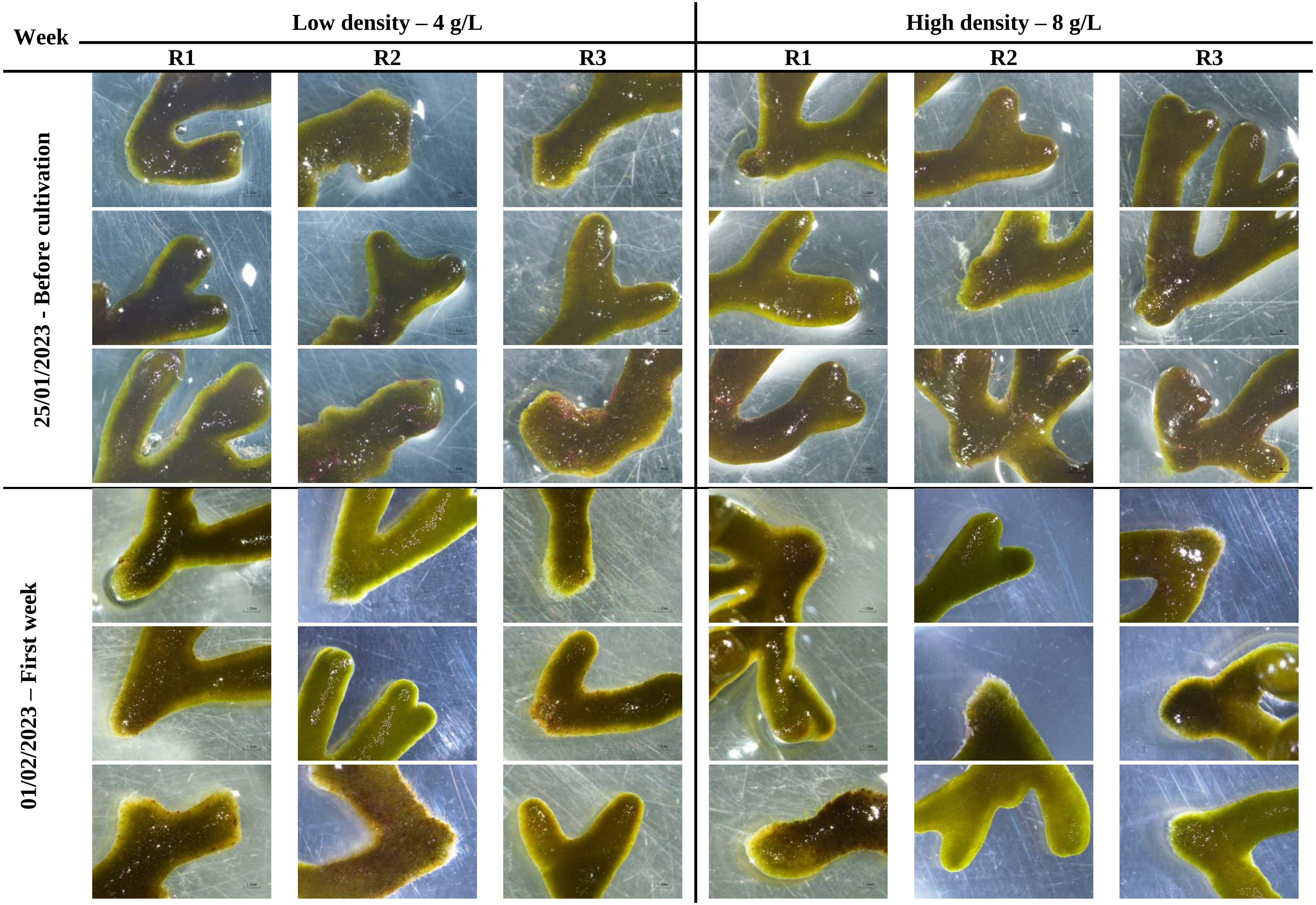
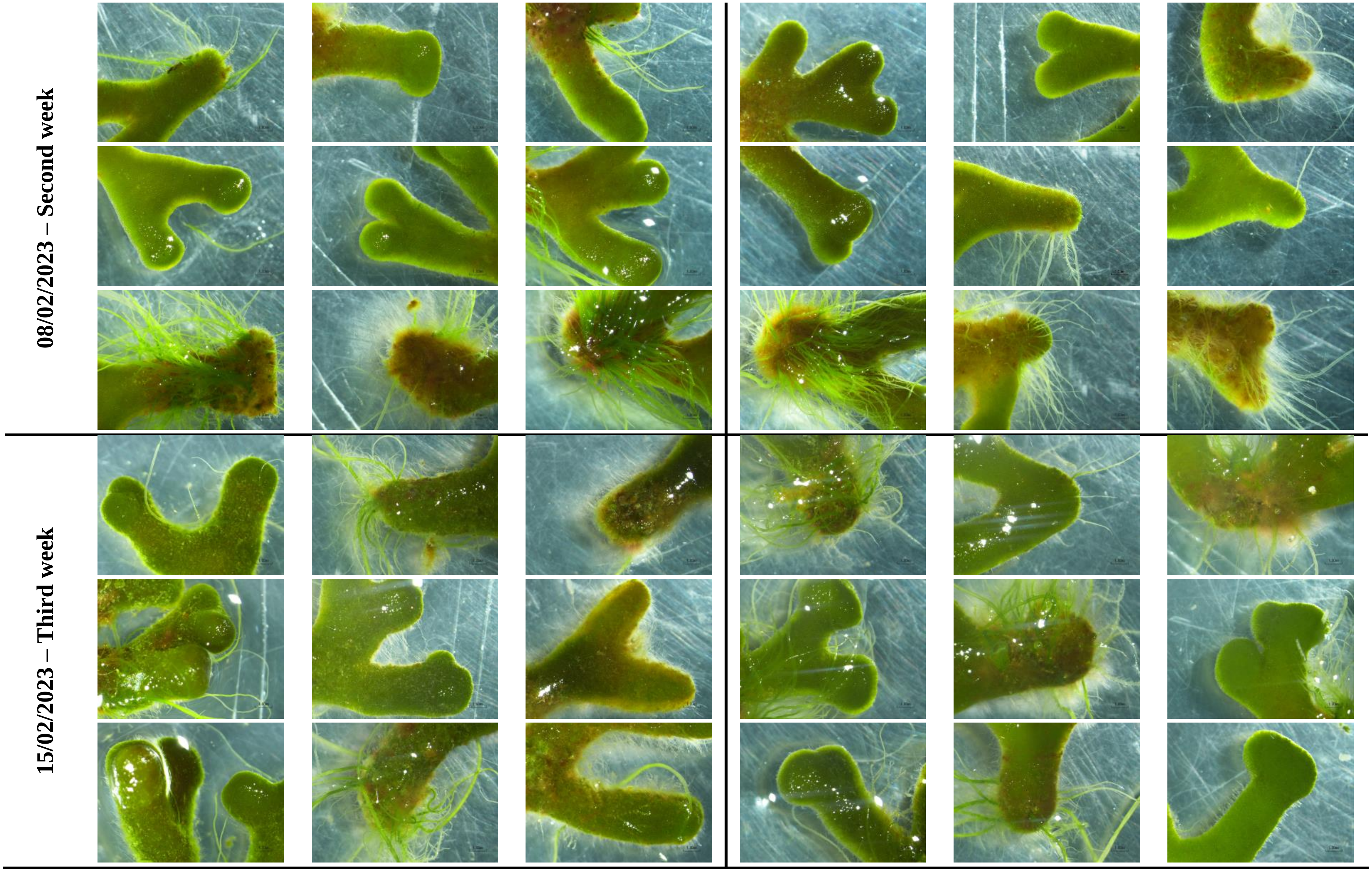
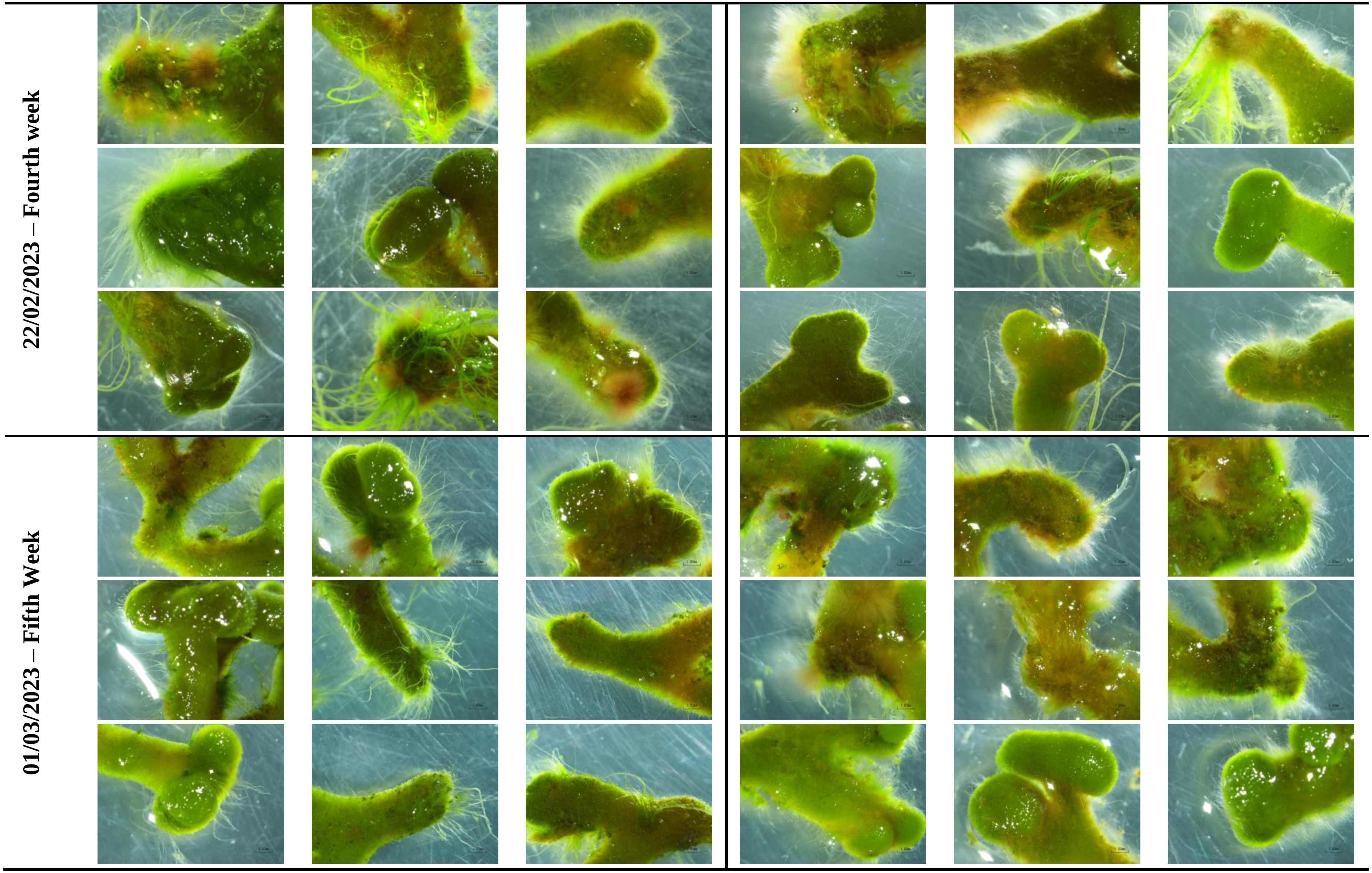
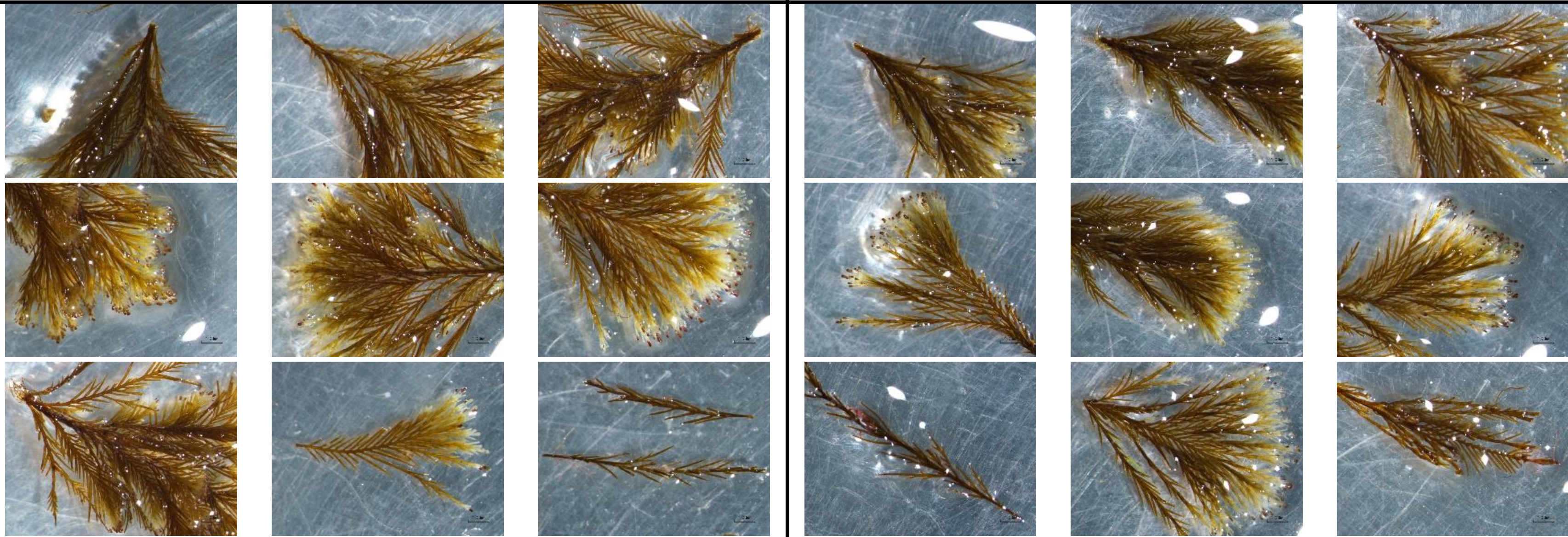
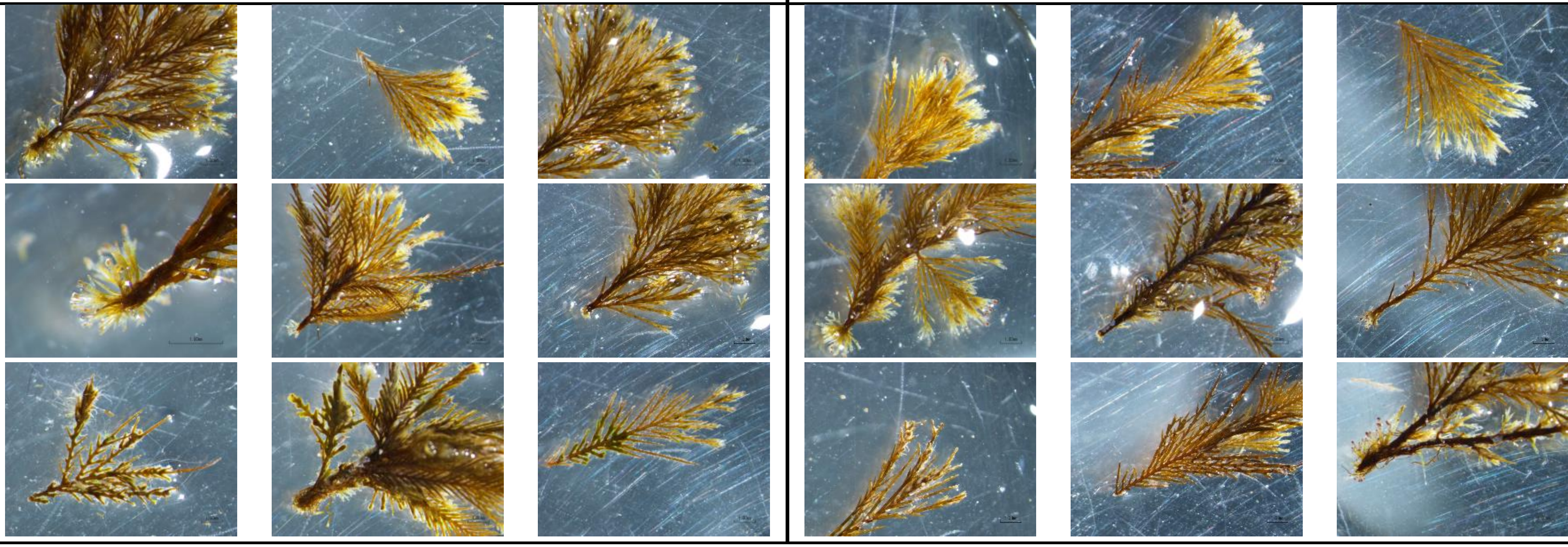
Annex 1 – *Codium tomentosum* tumble cultivation

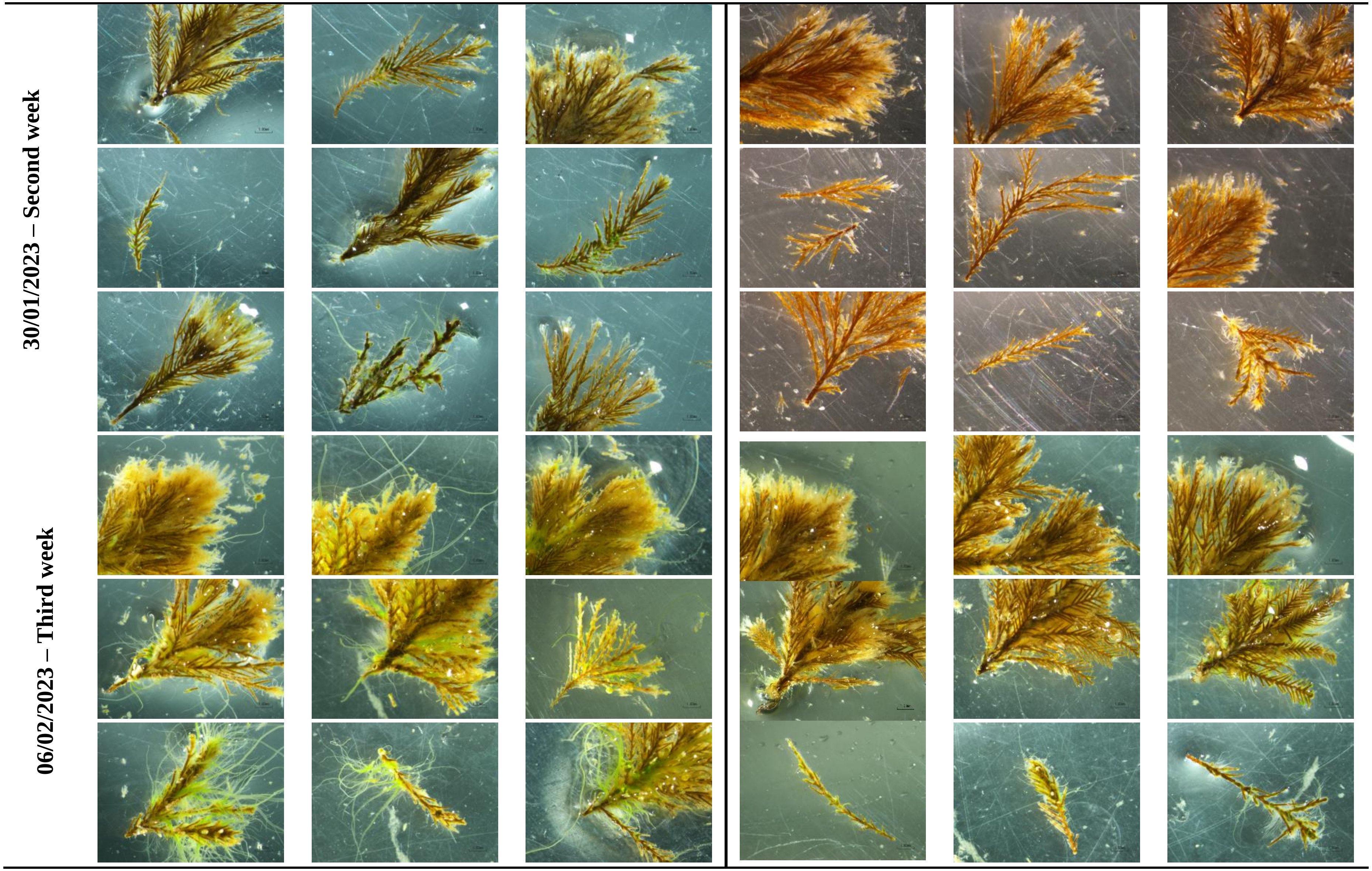


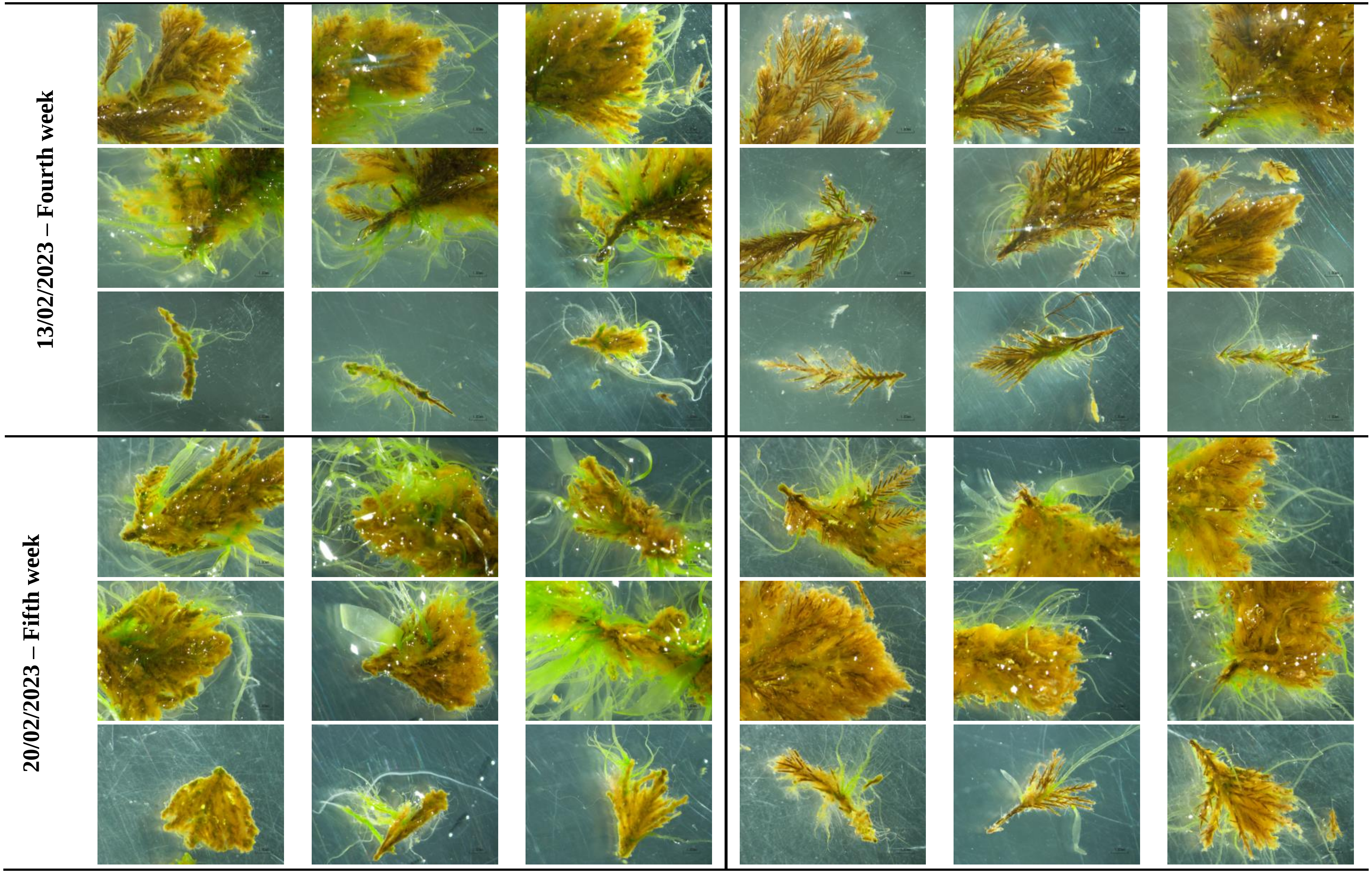

08/02/2023 – Second week
15/02/2023 – Third week


22/02/2023 – Fourth week
01/03/2023 – Fifth Week

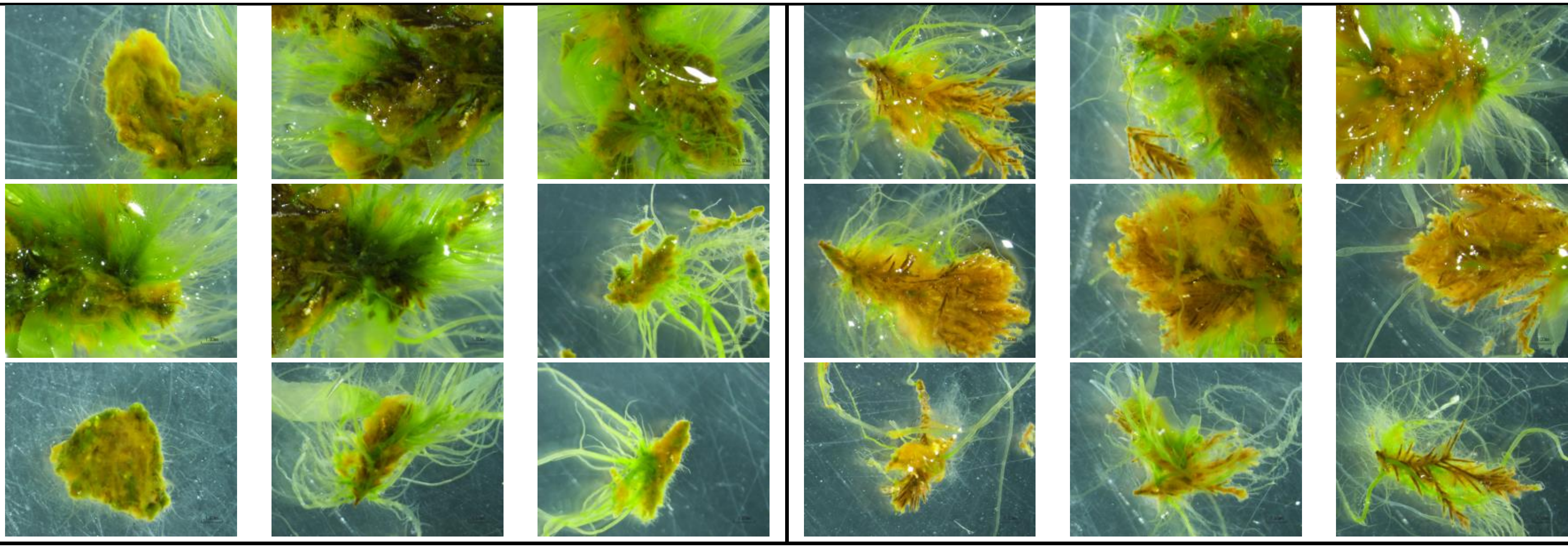
Annex 2 – *Halopteris scoparia* tumble cultivation

Week	Low density – 2 g/L			High density – 4 g/L		
	R1	R2	R3	R1	R2	R3
16/01/2023 - Before cultivation						
23/01/2023 – First week						


30/01/2023 – Second week
06/02/2023 – Third week


13/02/2023 – Fourth week
20/02/2023 – Fifth week

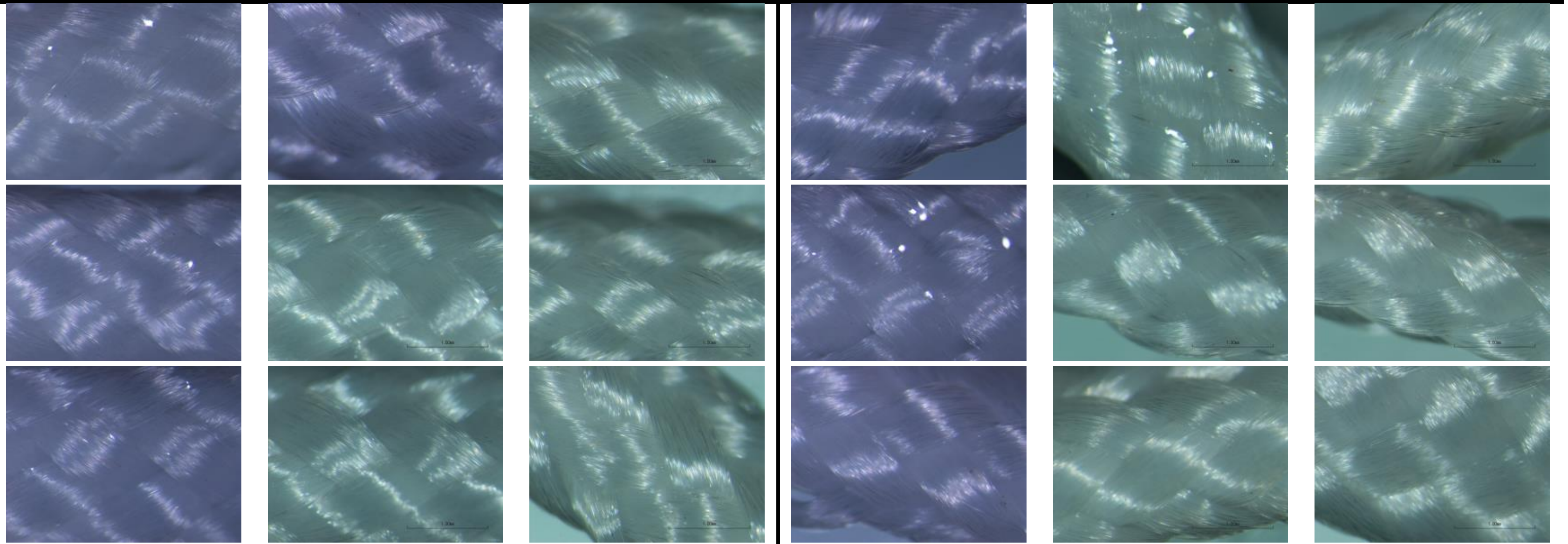
27/02/2023 – Sixth week



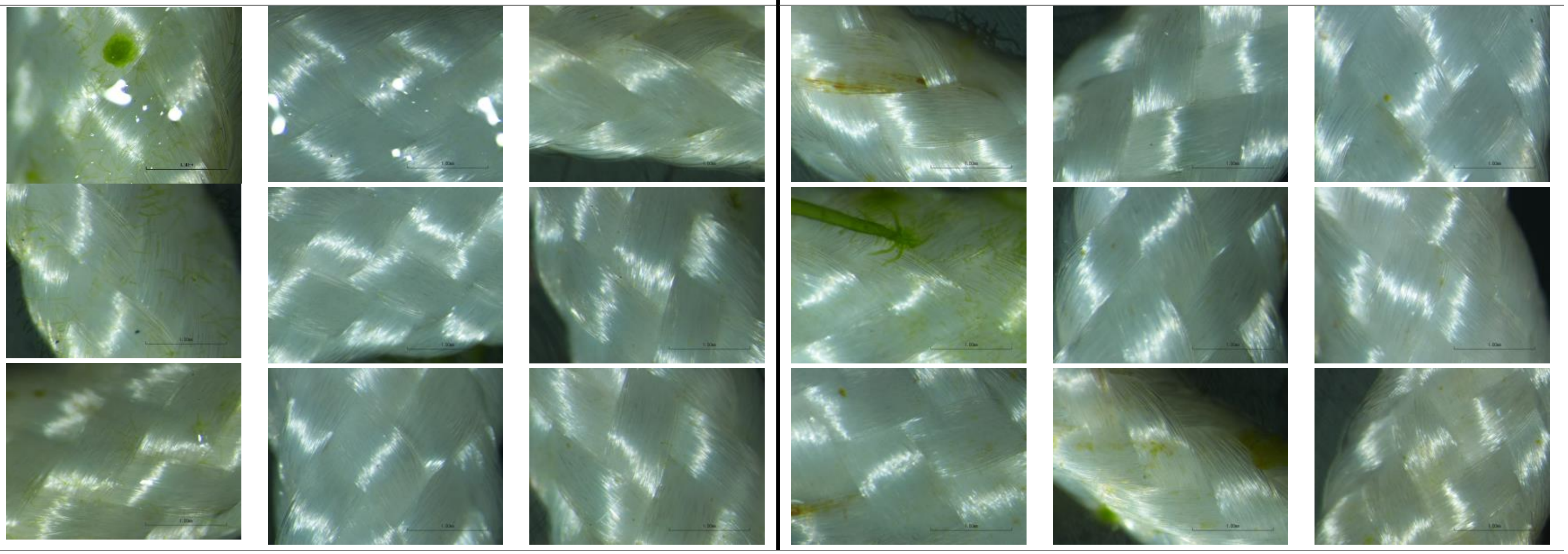
Annex 3 – *Codium tomentosum* substrate cultivation

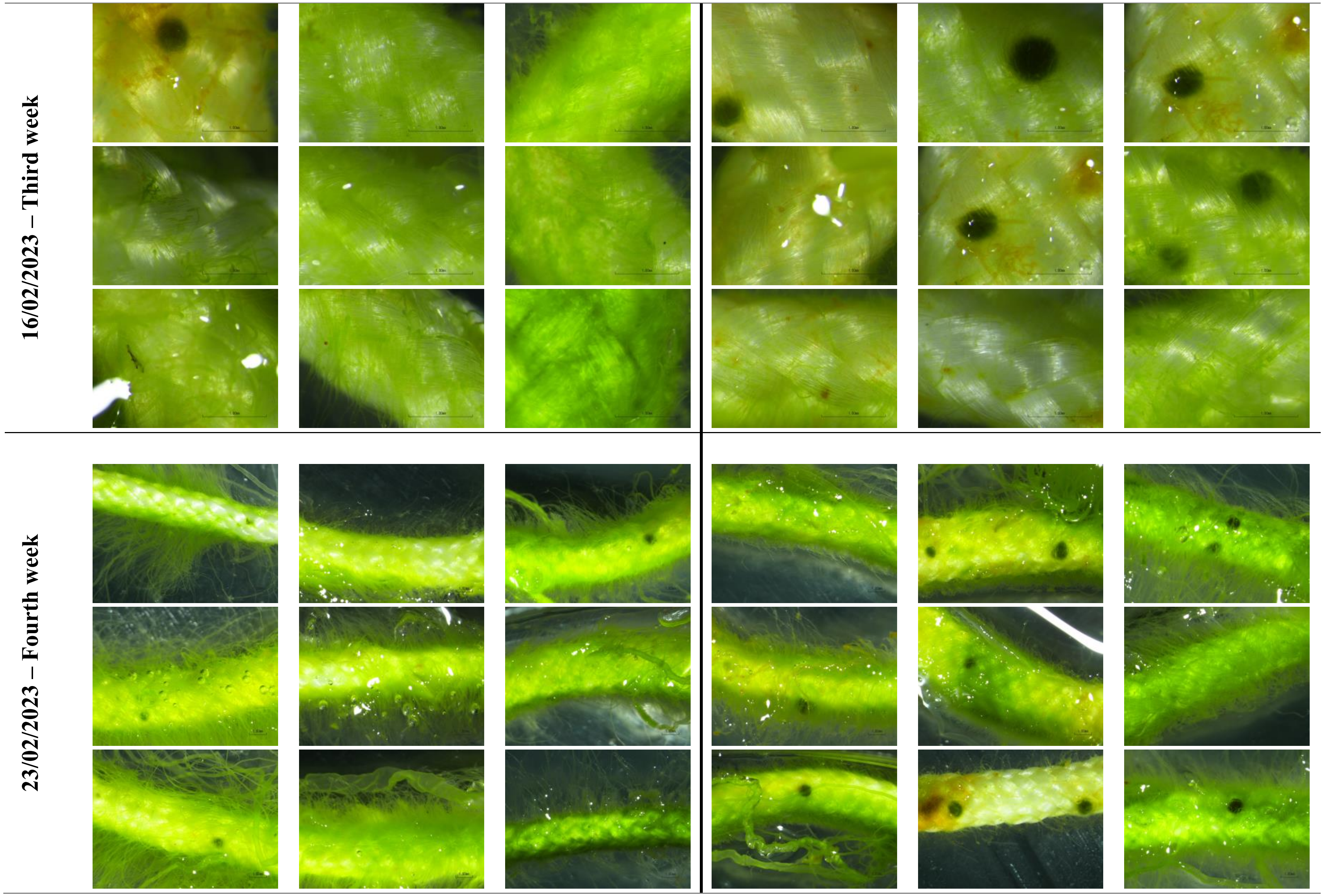
Week	Low density - 33 g/L			High density – 65 g/L		
	R1	R2	R3	R1	R2	R3

02/02/2023 – First week

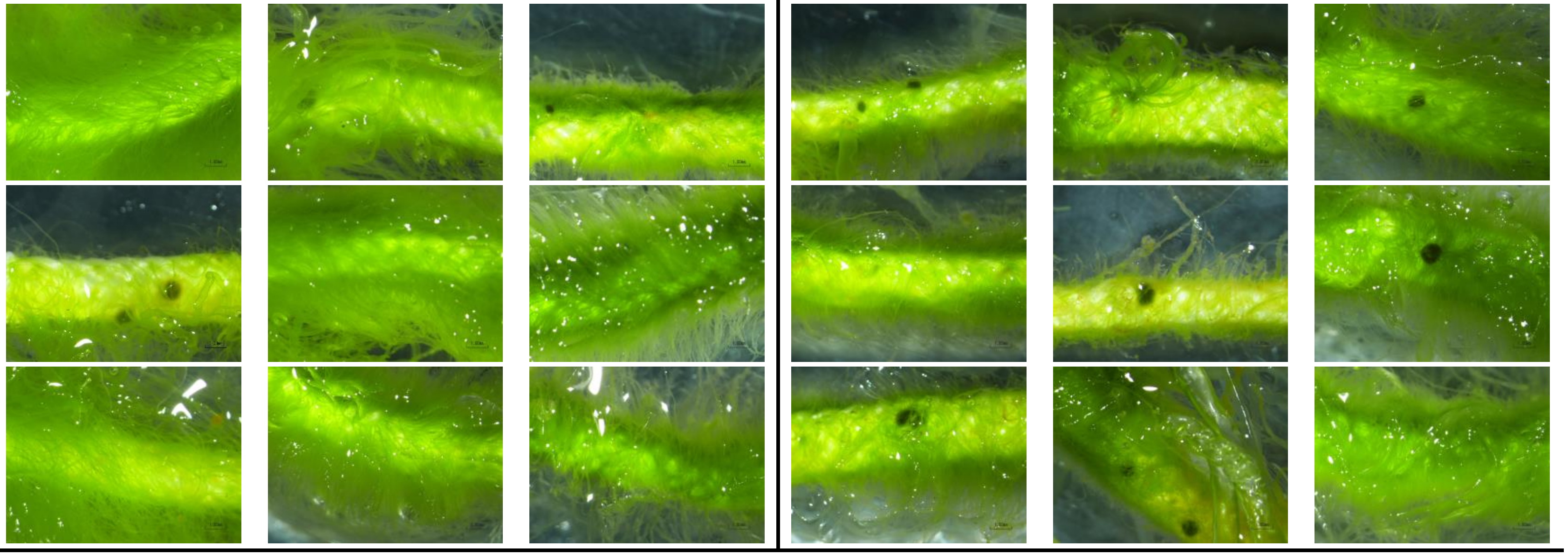


09/02/2023 – Second week

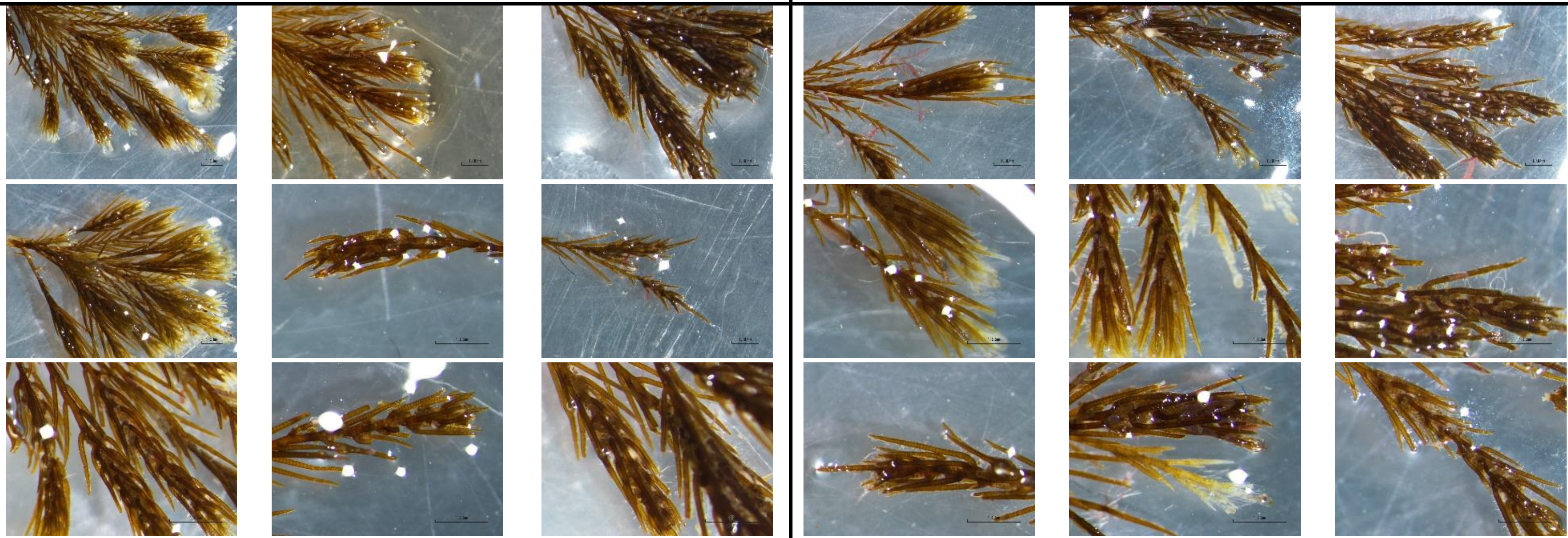
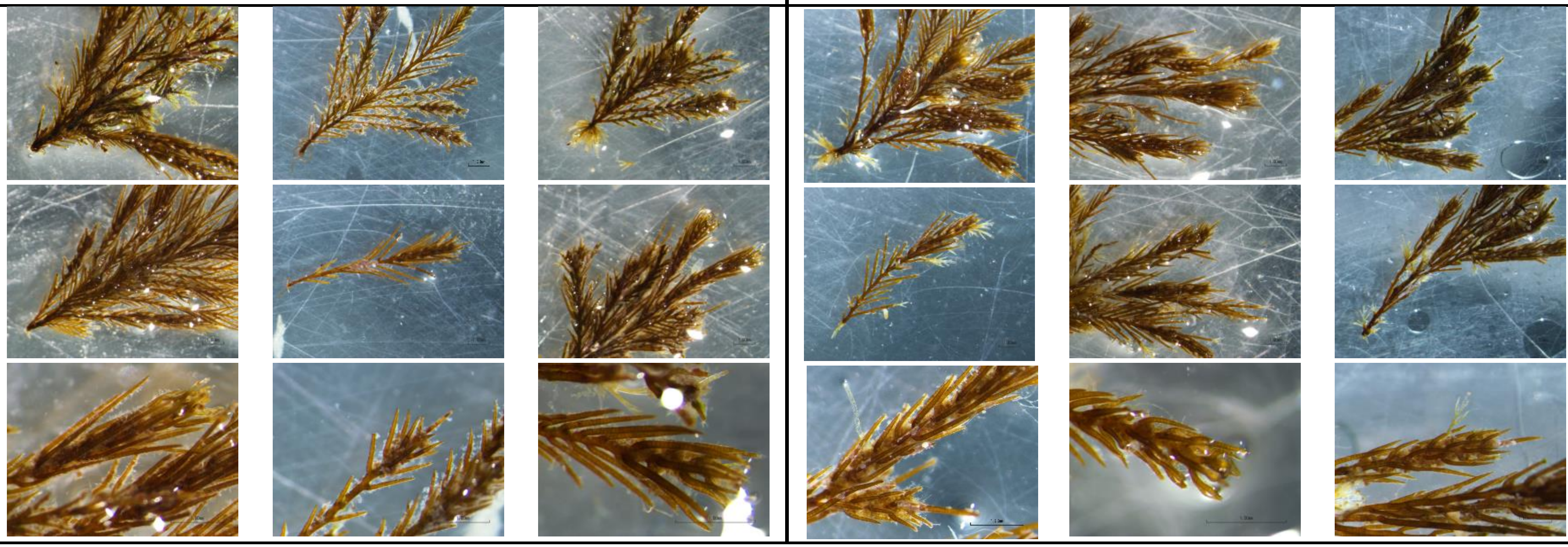



16/02/2023 – Third week
23/02/2023 – Fourth week

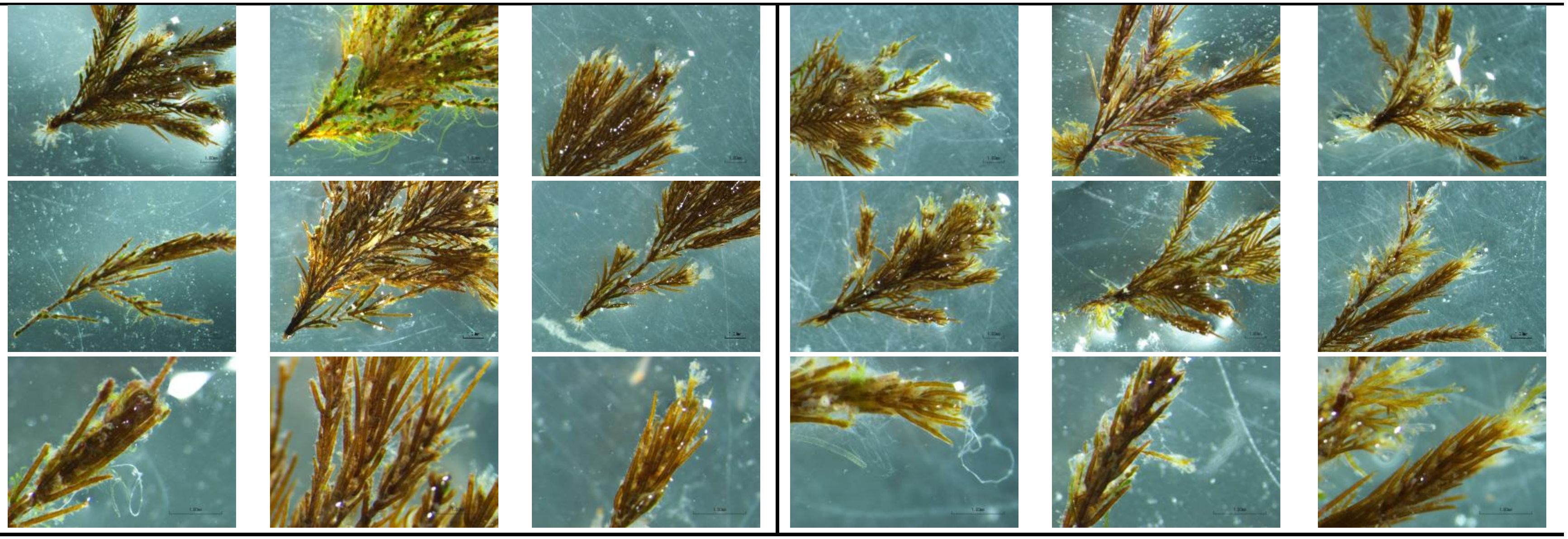
02/03/2023 – Fifth week



Annex 4 – *Halopteris scoparia* substrate cultivation macroalgae

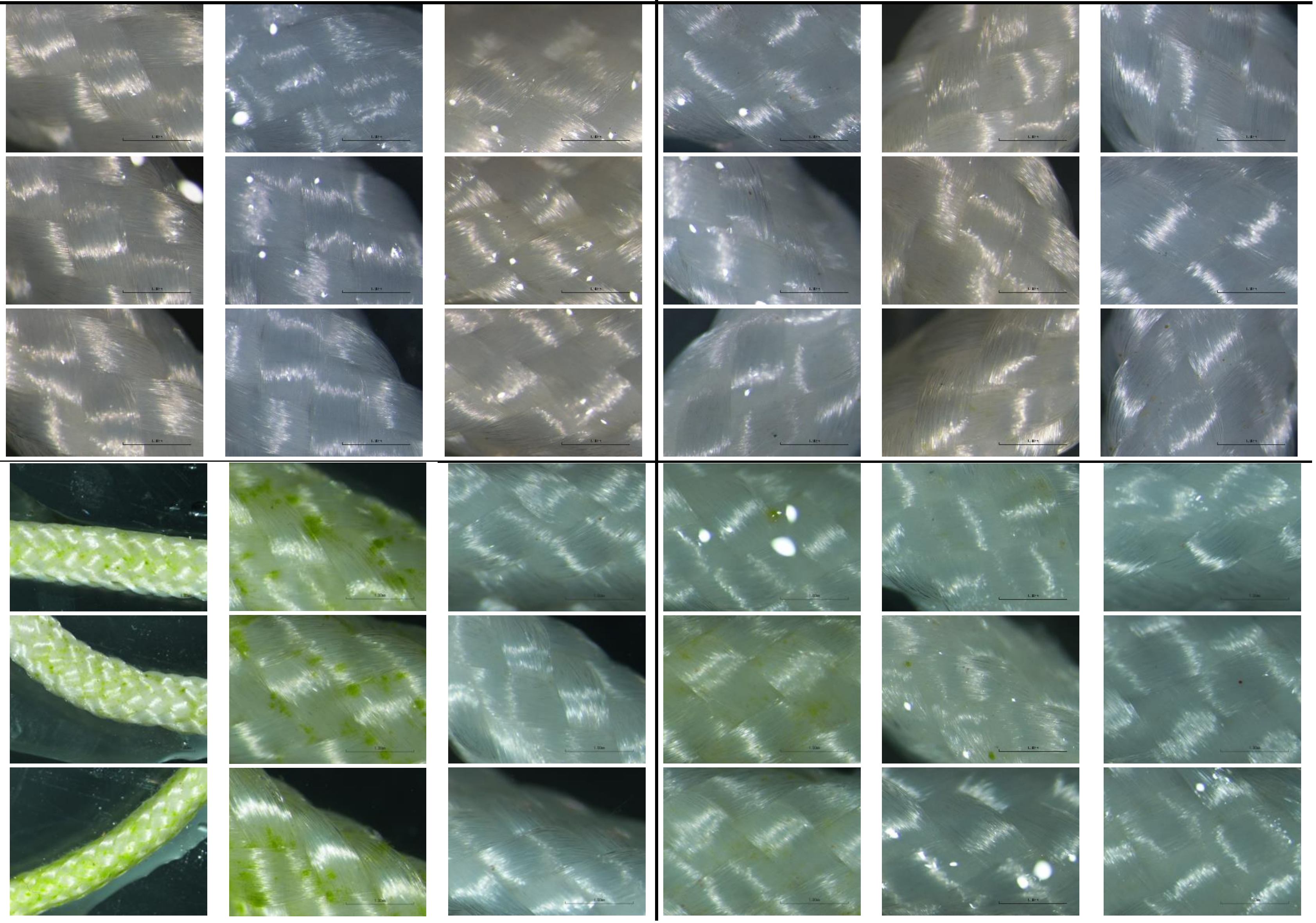
Week	Low density – 2 g/L			High density – 4 g/L		
	R1	R2	R3	R1	R2	R3
16/01/2023 – Initial biomass						
23/01/2023 – Frst week						

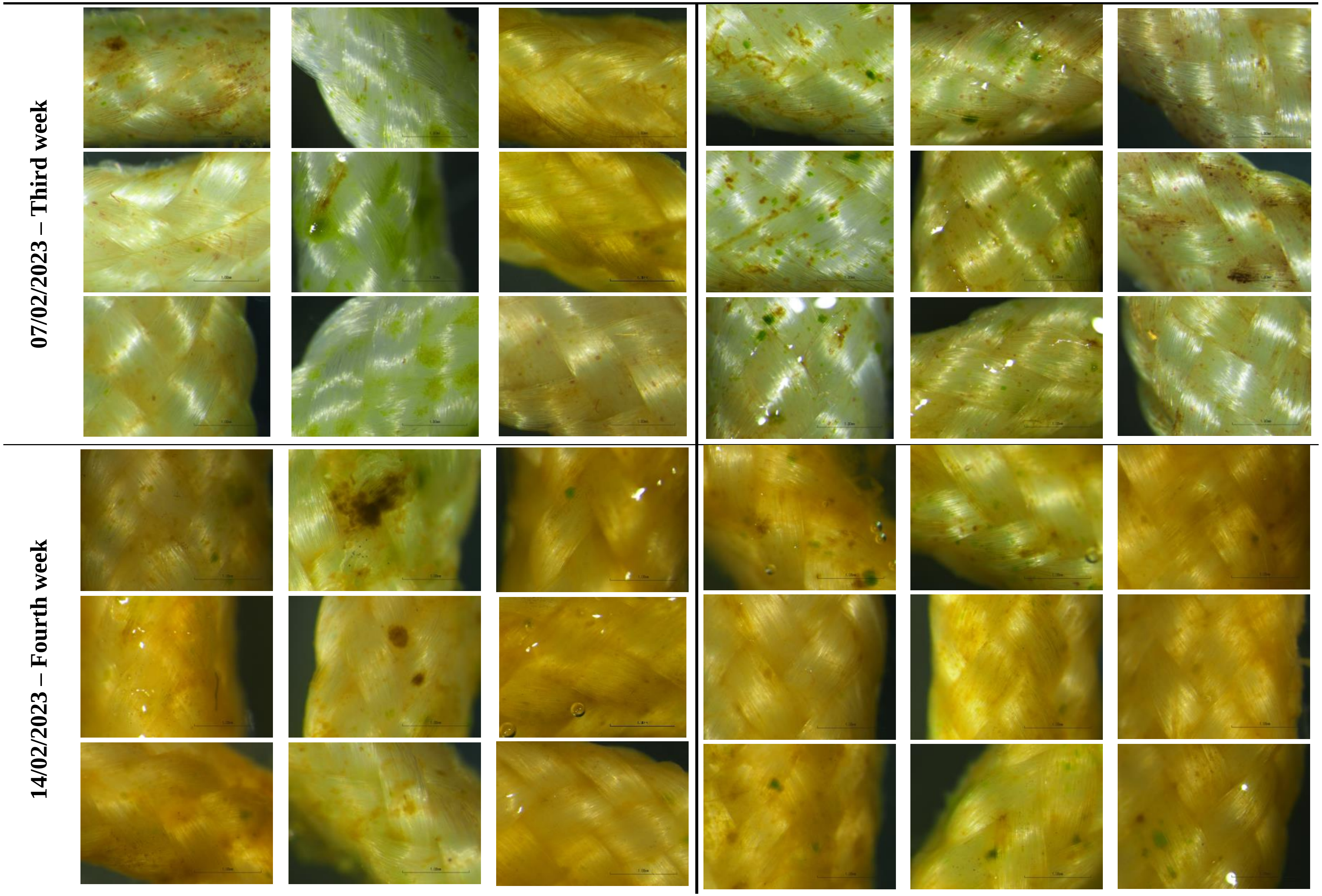
31/01/2023 – Second week



Annex 5 – *Halopteris scoparia* substrate cultivation

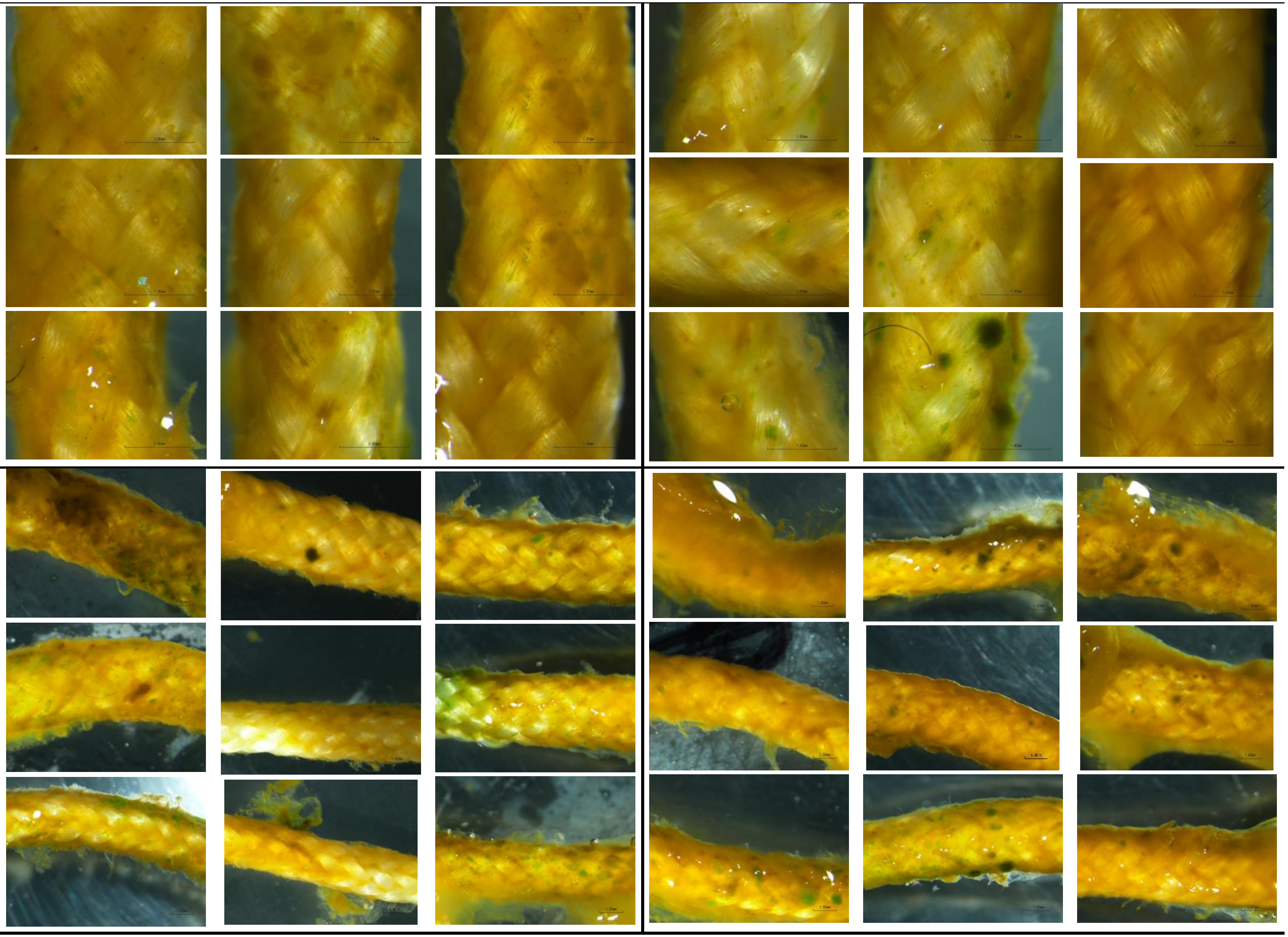
Week	Low density – 2 g/L			High density – 4 g/L		
	R1	R2	R3	R1	R2	R3
24/01/2023 – First week						
31/01/2023 – Second week						




07/02/2023 – Third week
14/02/2023 – Fourth week

21/02/2023 – Fifth Week

28/02/2023 – Sixth week



Annex 6 – *Halopteris scoparia* IMTA cultivation

Density / week	22/04/2023 – Third week		
5 g/L			
10 g/L			
15 g/L			
20 g/L			

Density / week	06/05/2023 – Fifth week		
5 g/L			
10 g/L			
15 g/L			
20 g/L			

Density / week	13/05/2023 – Sixth week		
5 g/L			
10 g/L			
15 g/L			
20 g/L			

Density / week	**20/05/2023 – Seventh week**		
5 g/L			
10 g/L			
15 g/L			
20 g/L			

Density / week	27/05/2023 – Eighth week		
5 g/L			
10 g/L			
15 g/L			
20 g/L			

Density / week	03/06/2023 – Ninth week		
5 g/L			
10 g/L			
15 g/L			
20 g/L