Improving the elemental composition of *Gracilaria vermiculophylla* taking advantage of rapid ecophysiological response to salinity

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Internship Report to obtain the Master's Degree in Biotechnology of Marine Resources

Internship carried out under the supervision of Liam Morrison
and co-supervision of Ricardo Bermejo and Clélia Afonso

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Abstract

This work is part of an internship held in a project developed by the Earth and Ocean Science Department of the Ryan Institute at the University of Galway (Ireland) called MACROMAN, which is required to complete the Master's Degree in Marine Resources Biotechnology of the School of Tourism and Marine Technology of the Polytechnic University of Leiria.

Macroalgal blooms have been well recognized as an environmental problem since the 1970s. These blooms refer to the rapid and excessive growth of macroalgae in aquatic ecosystems, typically in marine environments, and they contain large amounts of biocompounds which make them of great interest for different human applications. The MACROMAN project focuses on the understanding of the mechanisms behind the development of these macroalgal blooms, to develop appropriate management tools. The main goal of this internship was to develop an experiment with one of the most important bloom-forming macroalgae in the Irish estuaries, *Gracilaria vermiculophylla*, and to carry out research about the improvement of its elemental composition, taking advantage of rapid ecophysiological responses to salinity. Small amounts of *G. vermiculophylla* were subjected to short incubation protocols at four different salinities and nine different incubation times, to analyse the fresh weight variation, water content and focus on the elemental composition of the seaweed tissue to enhance the concentration of desirable elements (e.g. K, N and P), and reduce the content of undesirable elements (e.g. Na, As, Cd, Co, Cu, Cr, Hg, Ni, Pb, Sn, Zn) for a future use in human applications (e.g. fertilisers).

This internship aimed to enhance the theoretical knowledge gained through the academic training, cultivate professional competencies, and offer insight into career opportunities within the job market.

Keywords: MACROMAN, Macroalgal blooms, *Gracilaria vermiculophylla*, salinity, ecophysiological responses.

Resumo

Foi realizado um estágio num projeto desenvolvido pelo departamento de Ciências da Terra e dos Oceanos do Ryan Institute da Universidade de Galway (Irlanda) denominado MACROMAN, necessário para a conclusão do Mestrado em Biotecnologia dos Recursos Marinhos da Escola Superior de Turismo e Tecnologia Marítima do Politécnico de Leiria.

Os blooms de macroalgas têm sido reconhecidos como um problema ambiental desde a década de 1970. Estes blooms referem-se a um crescimento rápido e excessivo de macroalgas em ecossistemas aquáticos, tipicamente em ambientes marinhos, e contêm grandes quantidades de biocompostos que as tornam de grande interesse para diferentes aplicações humanas. O projeto MACROMAN centra-se na compreensão dos mecanismos subjacentes ao desenvolvimento destes blooms de macroalgas, para desenvolver ferramentas de gestão adequadas. O principal objetivo deste estágio foi desenvolver uma experiência com uma das mais importantes macroalgas formadoras de blooms nos estuários irlandeses, a *Gracilaria vermiculophylla*, e realizar investigação sobre a melhoria da sua composição elementar, tirando partido das rápidas respostas ecofisiológicas à salinidade. Pequenas quantidades da *G. vermiculophylla* foram submetidas a curtos protocolos de incubação, com quatro salinidades diferentes e nove tempos de incubação diferentes, para analisar a variação do peso fresco, o teor de água e a composição elementar do tecido das algas, com o objetivo de aumentar a concentração de elementos desejáveis (e.g. K, N and P) e reduzir o teor de elementos não desejáveis (e.g. Na, As, Cd, Co, Cu, Cr, Hg, Ni, Pb, Sn, Zn) para uma futura utilização em aplicações humanas (por exemplo, fertilizantes).

Este estágio teve como objetivo reforçar os conhecimentos teóricos adquiridos através da formação académica, cultivar as competências profissionais e oferecer uma visão das oportunidades de carreira no mercado de trabalho.

Palavras-chave: MACROMAN, blooms de macroalgas, Gracilaria vermiculophylla, salinidade, respostas ecofisiológicas.

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Lists of abbreviations, acronyms, and symbols

ASW – *Artificial Seawater*

ATP - *Adenosine triphosphate*

DGR – *Dividend Growth Rate*

DW – *Dry Weight*

FW_f – *Final Fresh Weight*

FW_o – *Initial Fresh Weight*

FWV (%) – *Percentage of Fresh weight variation*

GR (%) – *Percentage of Growth Rate*

HEPA - *High-efficiency particulate air*

MCPA - *4-Chloro-2-methylphenoxyacetic acid*

PAM – *Pulse-amplitude modulated.*

PE – *Peroxidase*

PPE - *Personal Protective Equipment*

SOP - *Standard Operating Procedure*

WC (%) – *Percentage of Water content*

1. Introduction

1.1 The Ryan Institute and the MACROMAN Project

The Ryan Institute is the largest research institute at the University of Galway, advancing sustainability and innovation with its Research Centers/Clusters and Research Groups (Fig. 1.1). The Ryan Institute's activities focus on sustainability research and innovation on four thematic research areas, namely Marine & Coastal, Energy & Climate Change, Agriculture & BioEconomy, and Environment & Health **(University of Galway, n.d.)**. The Centre of Ocean & Exploration is one of the 12 Research Centers/Clusters spanning these four thematic research areas, and which includes the MACROMAN Project.

Figure 1.1: *Logo of the Ryan Institute and National University of Ireland, Galway.*

MACROMAN is an EPA funded project (Fig. 1.2) developed by the Earth and Ocean Science department of the Ryan Institute at the University of Galway, Ireland. This Project, led by Dr. Liam Morrison, aims to understand the mechanisms behind the occurrence and development of macroalgal blooms, in order to develop appropriate management tools. The main goal is to answer questions using a multi-disciplinary approach to this task, combining traditional and innovative methodologies including aerial survey techniques, molecular and biochemical analyses, and laboratory growth experiments **(Macroman Project, n.d.).**

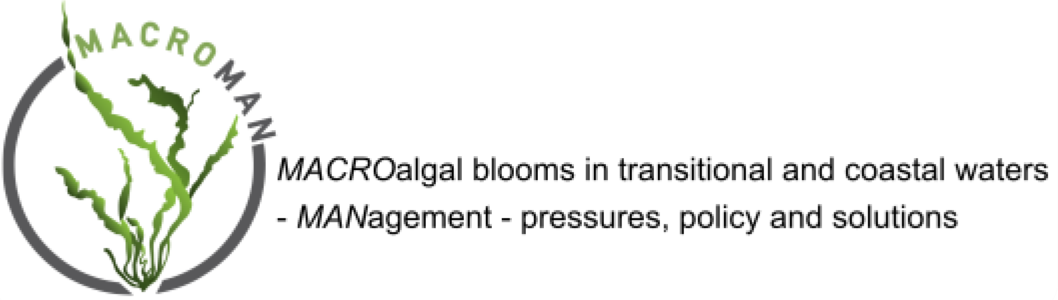

Figure 1.2: *Logo of the Macroman Project.*

The Irish estuaries (Fig. 1.3) affected by green, red, and brown seaweed blooms assessed during the development of the MACROMAN project were the Argideen and Clonakilty estuaries, Co. Cork, Tolka estuary, Co. Dublin and Killybegs estuary, Co. Donegal. This project seeks to identify the local environmental conditions explaining the occurrence and development of macroalgal blooms, to develop fast and inexpensive methodologies for the monitoring of macroalgal blooms and identify possible solutions and management actions. In order to address this aim, different laboratory experiments were developed to determine the ecological characteristics and tolerance levels of different bloom forming species for different environmental factors (e.g., salinity, temperature, light, nutrients…), and their putative implications on bloom formation in a global change context.

Figure 1.3: *Estuaries areas in Ireland where the MACROMAN project is focused on.*

The present internship was completed as part of this project at University of Galway, at the city of Galway, Ireland, and its main objectives were to improve practical skills in the area of marine biotechnology, putting into practice theoretical concepts learned during the 1st year of the Marine Resources Biotechnology master's degree. Among all the knowledge I gained, some stand out, such as the development of algae identification skills, cultivation, and maintenance of algae cultures in a laboratory environment, experimental design, preparation of analytical samples, and collection of specimens in the field, allowing the internship "Improving the elemental composition of *Gracilaria vermiculophylla* for their performance as biofertilizers, taking advantage of rapid ecophysiological response to salinity" to be carried out.

In addition, during this internship, I also took part in some other projects, which gave me knowledge about several subjects and areas in the marine biotechnology field, learning how to work and manage an assigned laboratory space, consequently, and working in research team.

I specifically chose to do this internship outside of Portugal, at the University of Galway, Ireland, because it is a leading institution, but also because it allowed me to improve my skills of social and professional integration and adaptation in a different country, as well as my language skills, having significantly improved my way of communicating in another language, in this case English.

1.2 Aims of the internship

This internship was held at the University of Galway, Ryan Institute, with the MACROMAN Project. The main aim of this internship was to gain practical experience in the laboratory work, such as the management of an assigned laboratory space, the use of different laboratory equipment, measurement of different physicochemical parameters of interest in seaweed culture, and laboratory procedures, such as protocols for the use of specific laboratories, acid bath cleaning, and the appropriately preparation of samples post experiments for analysis.

Furthermore, it was learned about the cultivation and maintenance of algal culture in a laboratory environment, which consequently leads to getting more knowledge about seaweed ecophysiology. The ability to prepare fieldwork for different sample collection was also one of the main objectives of this internship, together with the ability to work independently and in an international research team, fluency in English language and obtain experience in data analysis and scientific writing.

Finally, the main objective of this internship was to have the opportunity to develop and put into practice the project with the macroalgal blooms of *Gracilaria vermiculophylla* "*Improving the elemental composition of Gracilaria vermiculophylla taking advantage of rapid ecophysiological response to salinity"* (Figure 1.4).

Figure 1.4: *Seaweed* Gracilaria vermiculophylla*, while collecting samples for the experiment “Improving the elemental composition of* Gracilaria vermiculophylla*, taking advantage of rapid ecophysiological response to salinity.” at Roisin Bay, Galway.*

2. Internship Tasks Description

2.1 Laboratory Procedures

2.1.1 Management of an assigned laboratory space

Within the scope of a laboratory worker, the role of manager is to oversee the organization and use of the laboratory space. The manager has a range of responsibilities, from organizing workspaces, to ensuring the availability of resources, cleaning the space properly to avoid contamination, and contributing to the overall efficiency of the research activities.

In the MACROMAN Project, there was a main laboratory, where most of the experiments were carried out, a walk-in culture chamber, where the seaweed cultures were maintained, a cleanroom, which is an isolated, well-controlled and actively cleaned laboratory, and a chemistry laboratory, where most of the chemicals were stored and where the acid bath for cleaning specific materials was prepared. Each of these facilities followed different organization and cleaning protocols and conditions.

2.1.1.1 The Main Laboratory and the Chemistry Laboratory.

The main laboratory was the space that most of the experiments were conducted (Fig. 2.1). This facility was in turn shared with other departments, so the organization and cleanliness of the space not only involved the project in question, but also the work of others. For this reason, the process of disinfecting the space before and after use was very important, as was organizing each piece of equipment back into its proper place. To avoid confusion with the equipment and prevent the use of materials from other groups, some cupboards and benches were divided up for restricted use by the researchers on this project only.

Figure 2.1: *The main laboratory used from the research team of the Macroman Project.*

The use of acid baths in laboratories serves various purposes and is particularly important for certain applications, and this has been conducted in the chemistry laboratory. The primary role of the acid baths is to clean and decontaminate laboratory equipment, especially glassware and instruments, removing organic and inorganic residues. This helps to predominantly eliminate cross-contamination between experiments involving chemicals, biological samples, or complex solutions.

It is crucial to note that although acid baths are valuable tools in laboratories, proper safety precautions must be observed when handling acids. This includes using appropriate personal protective equipment (PPE), which includes gloves and lab coats int this case, working inside a fume hood, to limit the exposure to toxic fumes, vapours, or dust, and following stablished safety protocols. Additionally, the choice of the acid and the concentration, was of 10% of Hydrochloric Acid, which was required at this laboratory. At this project, the acid bath is part of a Standard Operating Procedure (SOP) to ensure consistency and compliance with best practices in laboratory hygiene.

2.1.1.2 Walk-in Culture Chamber

Culture chambers are specialized laboratory devices designed to create and maintain a controlled and customized environment for the optimum growth of organisms, in this case, seaweed species. Growing seaweeds requires specific conditions, such as temperature control, adequate light, nutrient availability, and water parameters. A walk-in culture chamber allows researchers or aquaculturists to create and maintain these conditions on a larger scale, increasing the likelihood of healthy growth.

The culture chamber consisted of several shelves, each shelf illuminated with two cool-white, fluorescent lamps, whit a 12:12 light/dark cycle provided by cool white LED lamps, whereby the lighting could be adjusted according to the samples present, and with an average temperature of 14º C.

2.1.1.3 Cleanroom

A cleanroom laboratory is a controlled environment designed to minimize the introduction, generation, and retention of contaminants. These contaminants may include airborne particles, dust, microbes, chemical vapours, all of which can impact sensitive processes such as biotechnical research, semiconductor manufacturing, pharmaceutical productions, and other precision scientific applications. Cleanrooms, in general, are characterized by stringent environmental control to ensure high levels of cleanliness and sterility.

This environment come with a cleanliness level quantified by the number of particles per cubic meter at a predetermined molecule measure. The air quality is also crucial for the efficiency of the cleanroom, and for that reason they have a sophisticated ventilation and air filtration system using High-efficiency particulate air (HEPA) to control particle levels. The cleanroom maintains precise control over the temperature and humidity levels to create a stable environment, with a temperature of 16º C.

As this is a very delicate environment, access is very restricted and controlled. Only people who had an authorization were allowed in with an access card. The entry point includes a gowning room where the personnel can change into cleanroom attire following protocols with specific procedures for entry and exit, such as:

- Disinfect footwear, gear, and hands at the entrance.
- The analyst should wear proper coats, face masks, hairnets, shoe covers and gloves, pass through adhesive mats before entering the clean room.
- The room should be always kept clean, which includes washing the material used before.
- Chemical samples should be kept inside the fume hood, to prevent the contamination in the air.

The cleanroom used by the MACROMAN project was composed with a few laboratories equipment such as a Laminar Airflow Hood, a Microwave Digestor, two Precision and Analytical Balances, one Refrigerator, one Freezer, a Desiccator cabinet, and a Milli-Q® Water Purification System.

2.2 Fieldwork

Fieldwork is an integral part of many research projects, providing the researchers with the opportunity to directly observe, measure, and interact with the phenomena they are studying. Involves the collection of firsthand data from natural settings or real-world environments to address research questions or objectives. In this internship the opportunity to participate in fieldworks for data collection has appeared several times.

All the materials were previously organized, such as a list of equipment required for the fieldwork, and a Risk Assessment had to be completed. A Risk Assessment is the process of identifying what hazards exists, or may appear in the workplace, how they may cause harm and to take steps to minimise harm.

The first fieldwork during this internship was to *Rusheen Bay, Galway, Ireland (53°15'34.0" N, 9°06'41.7" W),* with the purpose of *Gracilaria vermiculophylla* collection. The material needed for that work included, waders, a bucket, to place the seaweed, at least two nets, to wash the seaweed in situ, with seawater, and gloves.

Following, it was collected some *Fucus vesiculosus* in the *South Park Beach at The Claddagh, Galway, Ireland (53.263814 N, -9.052106 W)*, for another project. For this fieldwork it was used plastic bags to carry the seaweed, gloves, and wellies due to the sediment of the workplace. Coming back to the laboratory, a sorting process was carried out to remove fertile individuals that couldn't be useful for the respective experiment.

Subsequently, another fieldtrip was carried out at *Killybegs, Co. Donegal (54.649909 N, -8.425907 W)* to collect *Pilayella sp.*, where three replicates of fresh biomass were taken from twenty-two samplings points with latitude and longitude data, and its observations. The material used for this task included two squares with a specific area (cm^2), a GPS for the information of time, latitude and longitude, wet suits, gloves, wellies, and plastic bags respectively identified with the sampling points for the collection of fresh biomasses.

Finally, a fieldwork to collect eighteen samples of seagrasses *Zostera marina* was carried out from a sheltered, secluded bay near *Lettermore, Connemara, Western Ireland*. A wet suit, a snorkel and goggles were needed because each specimen was observed underwater to ensure the presence of three internodal nodules above the sheet. The specimens were carefully placed in damp tissue paper, and transported to a sealed plastic ziplock bag, and placed inside a protective cooler box for transportation to the laboratory.

2.3 Cultivation and Maintenance of Algal Culture in a Laboratory Environment

The cultivation and maintenance of algal cultures in a laboratory environment involve creating and sustaining conditions that support the growth and development of algae for various research, industrial and educational purposes. With that being said, it is the laboratory technician's responsibility to make sure that those conditions are favourable and stable.

A technician is responsible for the daily routines previously assigned by the technician supervisor. A checklist needs to be followed to organize the workflow of the facility. The checklist is slightly different according with the different tasks or projects in process at the moment, but a few tasks were needed to do at least once a day, such as, the addition of culture medium to the seaweed cultures, preparation of ASW, confirmation that the chamber conditions were correct, such as temperature, light intensity, and shaker speed, and also if the seaweed looked healthy with no contamination. Every three days it was also needed to change the water of every culture and every seven days the cultures were weighted to calculate the relative Daily Growth Rate (DGR).

It is in the technician's duty to give the cultures the medium and know how to prepare it when finished. The culture medium used for the culture was the F/2 Nutrient Solution, which is a commonly used general enriched seawater medium, required for growing coastal marine algae, especially diatoms **(Algae Research and Supply, n.d.).** The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half.

The production of ASW is also very important, and it was prepared at least 10L per day, depending how much it was used. In a bucket, it was weighted 371g of the salt (Coral Reef), diluted in 10L of distilled water, and left approximately ten minutes on a stirrer with a magnet to ensure the dilution. At the end the water was measured with a refractometer to make sure the water had 34‰.

2.4 Opportunity to Assist in different projects.

While completing my master's internship, I had the opportunity to contribute and collaborate on various projects beyond the primary focus on my research. These additional projects encompassed assessing the effects of herbicides – MCPA, Glyphosate, 2,4-D and Triclopyr on the biological performance (DGR, tissue N and P, Fv/Fm) of a bloom forming species *U. flexuosa,* and two macrophytes which are typical of pristine estuarine environments: the brown seaweed *Fucus vesiculosus*, and the seagrass *Zostera marina*. I also took part in the assistance of a desiccation experiment, where the tolerance of several blooms forming species to two different temperatures (22.5 and 27ºC) was observed under experimental conditions. The desiccation experiment was assembled with *Pilayella sp., Gracilaria vermiculophylla, Ulva compressa* and *Ectocarpus sp.*.

Afterwards, I also took part in another project testing different concentrations of NH_4^+ and PO_3^{4-} in different incubations with *Pilayella sp.*, where it was assisted preparing material, setting everything up for the experiments and preparing the amount of stock solution of NH_4^+ and PO_3^{4-} and ASW needed. Besides all this projects I also had the opportunity to learn more with specific research about protoplasts with *Ulva sp.*

Through my involvement in these diverse initiatives, I gained valuable experience in how to organize a work environment and experiments for research, how to organize fieldworks and the data collected from it, how to cultivate and maintain a seaweed culture, the opportunity to handle different equipment such as Pulse-amplitude modulated (PAM), a spectroradiometer, a microwave digester, further enhancing my overall research capabilities.

Learning how to organize my tasks and my timetables according to what was needed and gaining the ability to work independently with international research teams was one of the most important things. These experiences allowed me to engage with interdisciplinary teams and expand my expertise in the marine biotechnology research field.

2.5 Running my own experiment.

During my master's internship it was gave it to me the opportunity to design and execute an independent project that focused on the improvement of the elemental composition of the seaweed *Gracilaria vermiculophylla*, taking advantage of rapid ecophysiological response to different ranges of salinity. This project was motivated by the large amount of biomass due to the occurrence of macroalgal blooms in the Irish coast. In certain conditions, one of the management actions proposed is the harvesting of bloom biomass in order to prevent anoxic events. The harvested biomass is usually disposed for landfill, which suppose an extra cost to the removal of the biomass for the public administration. In order to reduce the cost of seaweed bloom management, the possibility to enhance biomass quality through short incubation protocols for further biotechnological applications was explore. Specifically, the initial plan was to explore the possibility to increase N and K contents, as these elements are of interest for the biostimulant and fertiliser industry (as these are considered limiting nutrients for agriculture). In the planning phase, I learned how to develop a comprehensive research plan, outlining the objectives, methodologies and expected outcomes.

3 **Experiment:** *Improving the elemental composition of Gracilaria vermiculophylla, taking advantage of rapid ecophysiological response to salinity.*

3.1 Introduction

3.1.1 Macroalgae

The 70% of the surface of our planet is covered by the ocean, which means that there is a vast source of resources and biodiversity (**Resende et al., 2022).** In the last few years, it was estimated by **Guiry, 2012**, that the number of living algae could range from 30,000 to 1 million distinct species. Generally, algae are considered to be aquatic, oxygen-evolving photosynthetic autotrophs, unicellular, colonial, build by filaments or composed of simple tissues **(Guiry, 2012).**

The term algae cover a range of organisms with different phylogenetic origins that can be categorized as multicellular macroalgae and unicellular microalgae **(Ariede et al., 2017),** belonging to three different kingdoms, namely, Plantae, Chromista and Bacteria. These organisms can be found on the ocean floor up to 180m but are often found in solid substrates at a depth between 0 and 40m **(Kumar et al., 2021).** They are photosynthetic organisms that generally thrive attached to rocks in coastal areas. These organisms are divided in four different groups distinguished on the basis of their predominant pigments, which are, blue-green (phylum Cyanobacteria), brown (phylum Ochrophyta), red (phylum Rhodophyta) and green (phylum Chlorophyta) seaweeds, containing fucoxanthin, chlorophyll a, phycocyanin and phycoerythrin, respectively **(Fook et al., 2017; Cavaco et al., 2021; Kumar et al., 2021).** Although they all have chlorophyll b, their names derive from the resulting colours their predominant pigments produce, along their structure.

As the marine environment can be unstable in terms of conditions, these marine organisms have adapted themselves to a range of physical parameters, such as changes in pH, salinity, temperature, pressure, nutrient availability, and sun exposure **(Pangestuti et al., 2018).**

The wide diversity in the biochemical composition of these organisms provides an excellent reservoir to explore functional materials and constitute good sources of nutrients, many of which are hard to find in other taxonomic groups and that’s why they have attracted special interest, especially as most of these characteristics are not found in terrestrial environments **(Alves et al., 2016; Pangestuti et al., 2018).**

3.1.2 Macroalgal Blooms

Coastal marine ecosystems are considered one of the most productive habitats in the world **(Lane-Medeiros et al., 2023).** However, it is also considered one of the most degraded, subject to environmental changes resulting from the human occupation and exploitation **(Crain et al., 2009; Lane-Medeiros et al., 2023)**. Eutrophication has been identified as one the most important pressures affecting aquatic ecosystems **(Hering et al., 2010; Glibert et al., 2017, 2020)**. Fertilizers and sewage discharges are among the main anthropogenic impacts that result in the eutrophication of these ecosystems (i.e., excessive nutrient enrichment) **(Malone & Newton, 2020; Wang et al., 2021).** The European Union defines cultural eutrophication as “*The enrichment of water by nutrients, especially compounds of nitrogen and phosphorus, causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the water balance of organisms present in the water and to the quality of the water concerned”*, **(European Commission, 1991)**. The illustrated threats depicted in Figure 3.1 pose significant challenges to coastal ecosystems by introducing elevated levels of nitrogen and phosphorus into marine environments. This nutrient influx results in areas experiencing nutrient over-enrichment, creating conditions conducive to increased macroalgae production, which in turn can lead to the formation of algal blooms. **(Smetacek & Zingone, 2013; Puppin-Gonçalves, 2020; Wang et al., 2021).**

Macroalgal blooms have been well recognized as an environmental problem since the 1970s **(Pihl et al., 1995; Smetacek & Zingone, 2013).** Macroalgal blooms are typically large-scale, ephemeral events, often involving monospecific blooms of native species that grow rapidly and can appear and disappear in one season, usually last less than a year **(Joniver et al., 2021).** Macroalgal blooms can contain native, non-native and/or invasive species **(Bermejo et al., 2020b; Joniver et al., 2021)**, and can occur in temperate and tropical waters, especially in estuaries and shallow coastal regions, where they are triggered by eutrophication, limited water circulation, low wave action and high temperature and irradiance **(Green & Fong, 2016; Joniver et al., 2021)**. They are not toxic in themselves, but the accumulation and consequent degradation of large amounts of macroalgae biomass can have negative consequences for the ecosystem and shore-based human activities **(Bermejo et al., 2020b)**, such as anoxic decomposition which can lead to the release of gaseous sulphur compounds (e.g. hydrogen sulphide, carbon disulphide, methyl sulphide). Exposure to these noxious gases can lead to health risks in both humans and wildlife and have a significant impact on biochemistry and biodiversity **(Wan et al., 2017)**.

Figure 3.1: *Harmful red macroalgae bloom.*
(https://classroom.sanibelseaschool.org/harmful-macroalgal-blooms)

While a significant portion of the literature concerning macroalgal blooms emphasizes their adverse impacts on individual species, communities, and ecological processes, it’s important to recognize that macroalgal blooms also yield several effects that can be deemed advantageous **(Lyons et al., 2014).** These phenomena enhance transfer of nutrients from the water column to the sediments and other macroalgae, consequently decreasing nutrient levels in eutrophic water **(Hardison et al., 2010; Lyons et al., 2014)**. Macroalgal blooms can also augment habitat complexity, promote dispersal of other species, and furnish animals with food and shelter **(Holmquist, 1994, 1997)**. As a result, in some ecosystems, the macroalgal blooms may actually increase, rather than reduce, biodiversity and secondary production **(Holmquist, 1997; Dolbeth et al., 2003; Lyons et al., 2014)**.

Besides the impact on human activities that can cause these blooms, other factors, such as temperature, light, and salinity, are also crucial in explaining the development of these blooms **(Gao et al., 2017; Bermejo et al., 2020b)**. This high amount of nutrients available, in combination with the arrival of exotic or invasive species that are able of forming blooms can increase the size and duration of these blooms **(Bermejo et al., 2019).**

3.1.2.1 Associated costs

Beyond that, the negative socio-economic aspects associated to the development of macroalgal blooms are often exacerbated by the scale of the algal biomass deposited. A previous study of **Charlier et al., 2008**, conducted in Brittany, France, reports that in 1992, 14,560m^3 of *Ulva* sp. seaweed were removed from the shoreline, at a cost of €1.8 million**.** Similarly, the removal of more than one million tonnes of green algal biomass in the Qingdao region of China during the 2008 *Ulva* blooms, cost €200 million **(Charlier et al., 2008).** Another study estimating the economic costs of the macroalgal blooms in the Canadian Lake Erie Basin, s suggest that algal blooms will impose equivalent annual costs equal to $272 million (Canadian dollars) in 2015 prices over a 30-year period if left unchecked, which the largest market cost will be imposed on the tourism industry with $110 million (Canadian dollars) in equivalent annual costs **(Smith et al.,**

2019). As it shows, macroalgal blooms can pose a threat for economic activities such as tourisms or aquaculture, and the valorisation of these biomass has been suggested that could bring revenue to the local economy **(Wan et al., 2017).**

Despite these challenges, macroalgae, have a long history of use in various human applications, including medicinal, nutritional, and industrial purposes **(Levine, 2016)**. There is potential to convert nuisance macroalgae into a useful resource, such as biofuels, through biorefining **(Joniver et al., 2021)**, for animal feeds, fertilizers, and pharmaceuticals, among other uses **(Wan et al., 2017).** Algal biotechnology offers a range of potential uses, including in wastewater treatment, food and feed production, energy generation, and the production of chemicals and pharmaceuticals **(Cannell, 1990).** There is a growing interest in the use of algal products in pharmaceutical, cosmetic, and public health applications, with a focus on the bioprospection of bioactive compounds **(Marinho-Soriano et al., 2012).**

3.1.3 Rhodophyta

Red seaweeds, phylum Rhodophyta, are one of the oldest groups of the eukaryotic algae, having almost 7000 recognized species. They possess a wide variety of different pigments such as, phycoerythrin, phycocyanin, chlorophyll *a* and carotene, and their cell wall is composed of cellulose, carrageenan, and agar **(Afonso et al., 2021).**

With their large richness of bioactive compounds, these seaweeds have potential fungal, antibacterial, antioxidant and antiviral activities with potential for industrial application **(Gates, 2010; Venugoupal, 2008; Cavaco et al., 2021).** They exhibit beneficial performances for human applications in a lot of different sectors, and according to Food and Agriculture Organization of the United Nations **(FAO, 2018)**, the organisms that possesses these biocompounds have been utilized throughout the world for centuries, not only for the food industry, but in various other industries, such as, the pharmaceutical, cosmetic, food and agriculture industry. They contribute greatly to the communities due to their rich composition of macrominerals such as sodium, calcium, magnesium, potassium, chlorine, sulphur, and phosphorus **(Cherry et al., 2019).**

3.1.3.1 Gracilaria vermiculophylla

Gracilaria vermiculophylla (Ohmi) Papenfuss 1967 **(Guiry & Guiry, 2023)**, represented in figure 3.2, is a cartilaginous and cylindrical, native from the East coast of Asia, which has been used for agar production and food **(Rueness, 2005; Thomsen et al., 2006).**

Figure 3.2: *Gracilaria vermiculophylla**, Barba, I. 2008.** source: AlgaeBase.*

This species demonstrates high resistance to environmental stress, such as low salinities, low light conditions or high grazing pressures **(Nejrup & Pedersen, 2010; Nylund et al., 2011)** and has a high dispersal capacity throughout fragmentation **(Surget et al., 2017).** Due to its resistance to stress and high dispersion ability, this species has been able to thrive in intertidal estuarine environments of North America, Europe, and North of Africa **(Kim et al., 2010; Krueger-Hadfield et al., 2017b; Bermejo et al., 2020a)**, where is considered an invasive species. They can grow in mudflats, as it stays anchored to the substrate by the burial of its basal parts or attached to small pebbles or the shells of calcareous organisms **(Bermejo et al., 2020a)**.

Under eutrophic conditions, the relatively high growth rates of *G. vermiculophylla* and resistance to grazing **(Nejrup & Pedersen, 2010; Nylund et al., 2011)** allow the blooming of this species. The accumulation of large amounts of seaweed biomass led to hypoxic conditions and the release of toxic compounds, when it is degraded by bacterial action, producing deleterious effects on the functioning and services that estuarine ecosystems provide. This alien seaweed can also bloom in areas, where native seaweeds are not capable, modifying native biological assemblages and biogeochemical cycles in soft-sediment habitats **(Ramus et al., 2017; Bermejo et al., 2020a)**.

G. vermiculophylla interact with native fauna, creating habitats for various epiphytes and invertebrates. These interactions with other species, along with their role as a primary producer in this ecosystem, reveal that their absence or prevalence, have known impacts on the marine ecosystem balance **(Thomsen et al., 2007; Benitt et al., 2022).**

3.1.4 Ecophysiological response

Intertidal seaweeds are subjected to a land-sea based life, which leads to unstable conditions that can fluctuate dramatically over short spatial and temporal scales, which present a physiological challenge for them **(Fernández et al., 2015; Bermejo et al., 2020a).** A variety of studies on the ecology and physiology of *Gracilaria vermiculophylla*, elucidates their ability to develop ecophysiological responses to a variety of environment conditions that may imply morphological changes in its elemental and chemical composition **(Bermejo et al., 2020a; Kameyama et al., 2021).**

The salinity effect on the ecophysiological performance of seaweeds, has been described to outline the species-specific tolerance, and consequently, upper, and lower limits of survival, as well as mechanisms of acclimation **(Karsten, 2012)**. In a situation of salt stress conditions, seaweed cells are still in full contact with liquid water, with a diminished water potential, and different physiological parameters have been studied when the cells are on those conditions, including rate of survival, growth, photosynthesis, respiration, and reproduction **(Karsten, 2012)**.

3.1.4.1 Salinity and the Osmotic Acclimation

Salinity is one of the main stressors in intertidal estuarine environments. Species able to cope with wide salinity variability, show physiological mechanism to maintain a stable internal osmotic potential and osmotic homeostasis, by controlling concentrations of both inorganic ions and small organic osmolytes in a process that is called osmotic acclimation **(Nitschke & Stengel, 2013).** This process is important for the survival of seaweeds and a fundamental mechanism of salinity tolerance that conserves the stability of intracellular homeostasis resulting in an efficient functional state of the cells **(Karsten, 2012).**

This process is separated in two different phases (Fig. 3.3). Phase I is characterized by rapid changes in turgor pressure caused by an enormous inflow or outflow of water, following the osmotic gradient **(Karsten, 2012)** and phase II which, on the other hand, is characterized by being a slower phase, since it can last from several hours to two or three days **(Karsten et al., 1996b)**. It is the adjustment of the concentration of osmotically active compounds (i.e., osmolytes) to achieve new steady-state conditions **(Kirst, 1990; Karsten, 2012; Nitschke & Stengel, 2013).** The main ions involved in this process are the K^+, Na^+, Cl^-, sulphate, nitrate, and phosphate. Although the availability of Na^+ in small amounts is generally beneficial, a large amount of Na^+ ions over K^+ is not and can often be toxic to the cell **(Kirst, 1990; Maathuis & Amtmann, 1999).** This happens because both chemical compounds, Na^+ and K^+, have very similar physicochemical characteristics, which will imply competition between Na$^+$ at the transport sites for K$^+$ entry into the symplast and may result in K$^+$ deficiency. This competition between Na$^+$ and K$^+$ can also inhibit metabolic processes that crucially depend on K+ **(Maathuis & Amtmann, 1999).** That could occur because significantly influx of Na+ can cause considerable growth inhibition and in salt sensitive or glycophytic species, induce mild toxicity manifestations in salt-tolerant species, yet potentially confer advantages to halophytes **(Maathuis & Amtmann, 1999).** In contrast to the toxic properties of Na+ (and Cl-), K+ is fully compatible to metabolic activities **(Karsten, 2012)**, although there is still no explanation for this fact, and also plays vital roles in the cell: it balances cytoplasmic charge, activate key enzymes like those involved in pyruvate formation, and contributes to cell turgor, providing structural rigidity **(Maathuis & Amtmann, 1999).** Hence, as highlighted by numerous authors, a crucial factor in salinity tolerance lies in the ability to uphold a favorable cytosolic K+/Na+ ratio **(Yeo, 1998; Maathuis & Amtmann, 1999)**.

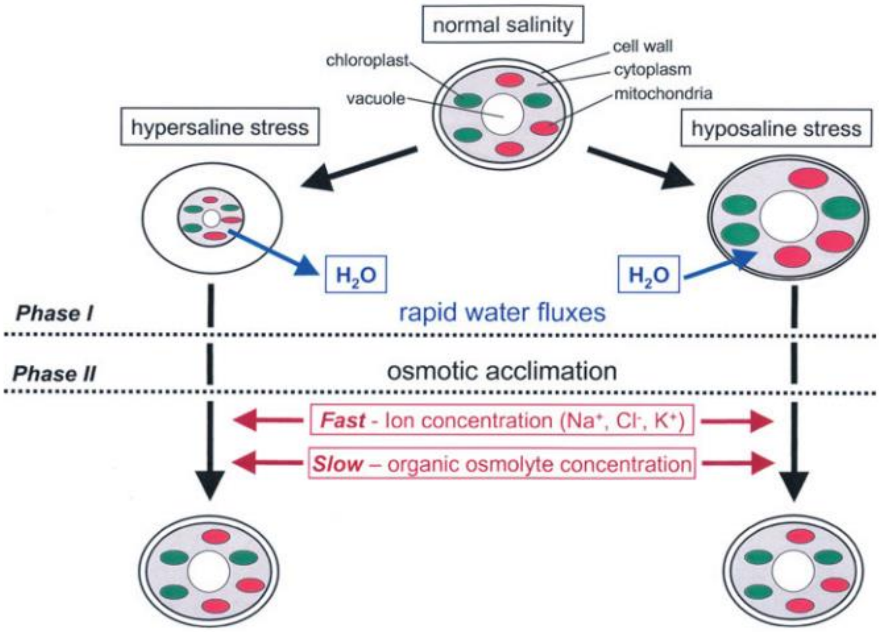

Figure 3.3**: Schematic summary of the processes of osmotic acclimation in seaweeds after hypersaline and hyposaline stress leading to a new steady state **(Karsten, 2012).

The water fluxes from Phase I are identified with rapid processes resulting in water influx under hyposaline conditions or water efflux during hypersaline stress, where both mitigate, at least transiently, the osmotic stress, lasting from minutes to hours **(Karsten, 2012).** Both Phase I and Phase II processes depend on the physicochemical properties of the cell-wall membrane complex, such as water permeability and water channels **(Karsten, 2012)**. A previous study from **Kirsten, 1990**, assumed that all phase I processes are not under metabolic control, but this was changed since the discovery of the so-called aquaporins, membrane-located water channels, that says that cells can control their water content **(Tornroth-Horsefield et al., 2006; Karsten, 2012).** These channels facilitate the transport of water across the lipophilic membrane. Probably, during salt stress conditions, many water channels at the cytoplasmic membrane open for short time and small water-soluble solutes are taken up or lost, during Phase I **(Karsten, 2012).**

3.1.5 Biomass optimization

Taking advantage of these ecophysiological responses, different attributes such as macrominerals contents (e.g., K, P, Mg or N), percentage of water, ash content, biocompounds concentrations, among others, can be manipulated to improve biomass quality and ensure product reliability, which is of interest for the seaweed industry. Therefore, to optimize incubation conditions, reducing time and space, the costs of the production, with the improve of the seaweed quality and simultaneously, the product reliability for human applications, it will be the key for profitability.

At present, marine macroalgae are emerging as prospective plant-based foods, often labeled as 'superfoods.' This term reflects their perceived health advantages attributed to their exceptional nutritional composition and abundance of bioactive phytochemicals **(Yuan et al., 2018**). The Na+/K+ ratio, a key indicator of this balance, is particularly relevant in the context of seaweed consumption. The Na/K ratio recommended by the World Health Organization (WHO) is close to one, so the intake of food products with this proportionality or below should be considered for healthy cardiovascular purposes **(Blaustein et al., 2012).** Research from **Circuncisão et al., 2018,** shows that the Na/K ratio in red seaweeds ranges between 0.1 to 1.8, which is the higher range compared to brown and green seaweeds. Likewise, particular high amounts of K have been described in red seaweeds, *Gracilaria* spp, and although seaweeds have a balanced Na/K ratio, their consumption could boost the K intake, however, their high Na content may elevate the overall Na intake in the diet, if salt replacement is not accounted for **(Circuncisão et al., 2018).**

Alternatively, enhancement of the mineral content in meat, fish, and other animal-derived products can be achieved through feeding animals with diets supplemented with algae **(Circuncisão et al., 2018).** There are several studies testing macroalgae as an alternative for animal food, and those research goes from a study by **Valente et al., 2015,** that tested three months of feeding rainbow trout (*Oncorhynchus mykiss*) with *Gracilaria vermiculophylla*-enriched fish meals (5%), to **Bikker et al., 2020**, that concluded that some of the red and green seaweed species (e.g. *P. palmata* and *U. lactuca*) would be a valuable protein source for farm animals. Seaweed is a sustainable feed source for livestock and aquaculture, with studies showing its positive effects on animal growth, product quality, and overall health **(Rajauria, 2015).**

The high production of K+ in this macroalgal blooms could be also extremely useful for the development of fertilizers. The need for K as a valuable fertilizer nutrient was demonstrated by Liebig's doctrine of mineral nutrition, leading to the commercial mining of K salts **(Darst, 2005).** Seaweed has long been recognized for its potential as a fertilizer due to its elevated levels of organic matter and essential nutrients, including potassium (K) **(Aitken, 1965).** Studies have shown that seaweed-based fertilizers can significantly enhance plant growth and yield, as well as improve soil properties such as pH and exchangeable calcium **(Zahid, 1999; López-Mosquera, 1997).** Furthermore, the application of seaweed sap (the liquid or extract derived from seaweeds), particularly from *Kappaphycus alvarezii* and *Gracilaria edulis*, has been found to increase wheat yield and nutrient content, with the highest concentrations of seaweed sap leading to the greatest improvements **(Shah, 2013).** These findings highlight the potential of seaweed-based fertilizers, particularly those rich in potassium, in sustainable agriculture.

Not only K is crucial for the development of fertilizers, but also the availability and application of nitrogen (N) as nutrients in soil and their role in yield development are decisive for the production of high-quality yields. The application of N and K fertilizers has been shown to increase herbage production and influence the botanical composition of pastures, with K being particularly important for the development and maintenance of white clover **(Mosquera-Losada, 2004).** The effects of N fertilization on crop quality have also been discussed, with N influencing the amino acid composition of protein and the quality of various crops **(Maheswari, 2017).** The molecular basis for N, P, and K uptake in plants has been reviewed, highlighting the importance of root system architecture and nutrient transporters **(Lyzenga, 2023).**

3.1.6 Main goals

The main goal of this study is to develop short culture protocols to manipulate the mineral composition of one species of seaweed (*Gracilaria vermiculophylla*) enhancing the concentration of desirable elements (e.g. K, P, N), and reducing the content of non-desirable elements (e.g. Na, Zn, Cu, Ni, Co, As, Cd, Cr, Hg, Pb, Sn), analysing as well its growth rate and percentages of carbon and hydrogen, using four different salinities (16‰, 32‰, 48‰ and 64‰) and nine different times of incubation (1min, 5min, 10min, 30min, 1h, 3h, 6h, 12h and 24h), to analyse the greatest combination of these conditions on the performance of the elemental composition levels. With this, the ultimate goal would be to find the best way to optimize this large amount of biomass caused by the macroalgal blooms, for human applications (e.g. fertilisers, human food and animal food).

3.2 Material and Methods

3.2.1 Seaweed collection and acclimation

Approximately 700 g of fresh *Gracilaria vermiculophylla* biomass was collected at Rusheen Bay, Galway, Ireland (53°15’34.0” N, 9°06’41.7” W), in November of 2021 (Fig. 3.4). The specimens were washed *in situ* with seawater using a net to remove the excess of sediment and transported to the laboratory in a closed cooler box where they were washed again, gently, with artificial seawater (ASW) to remove residues and epiphytes.

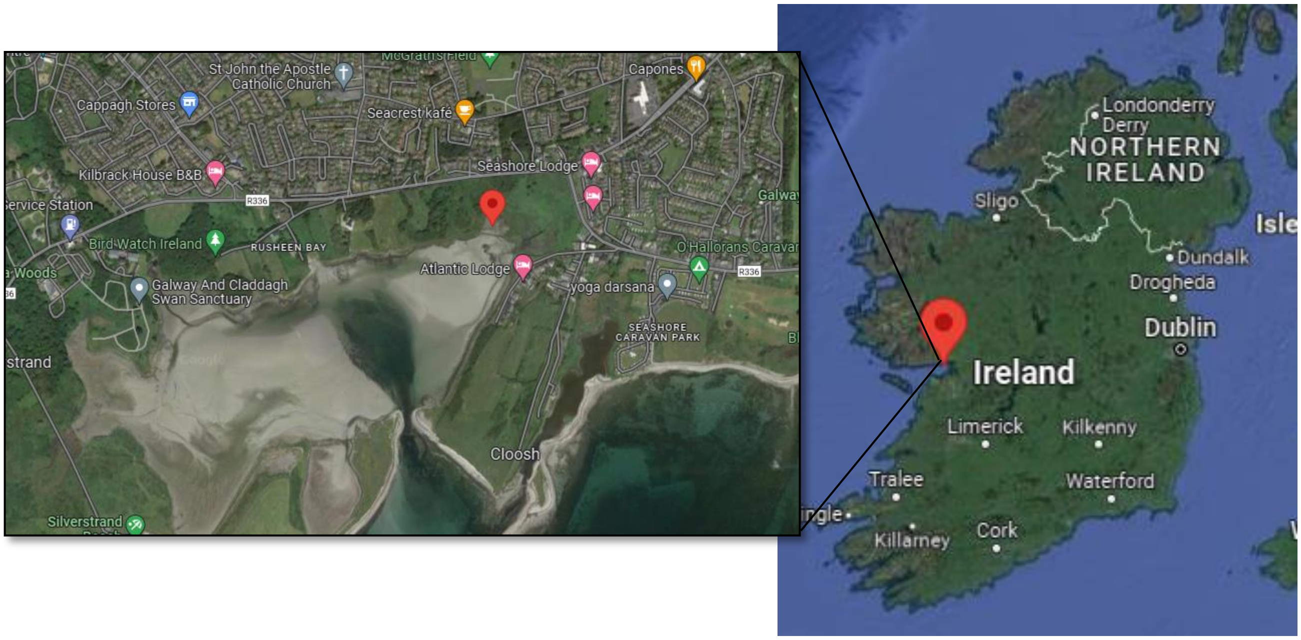

Figure 3.4: *Location of Rusheen Bay, in Galway, Ireland, where the biomass of Gracilaria vermiculophylla was collected.*

Subsequently, four aquaria were set up, containing 15L of artificial seawater each, at 32‰ of salinity, together with 175g of *G. vermiculophylla* biomass, for five days (Fig. 3.5). The replicates were placed in walk-in culture chamber, where temperature was set to 16°C, and illumination by cool-white, LED lamps at 90 µmol photons $m^{-1}s^{-1}$. Four air pumps were used inside each aquarium, to ensure water motion.

Figure 3.5: *Collection of G. vermiculophylla in Rusheen Bay, Galway.*

3.2.2 Experimental set-up

The experiment was planned to identify the best time and salinity conditions to increase the performance of the chemical composition of the *G. vermiculophylla* tissue, according to the most desired elements. That said, nine different incubation times and four different salinities, were designed to observe the difference in the seaweed tissue, between higher and lower salinities. For 36 treatments (9 incubations with 4 different salinities) (Fig. 3.6 and 3.7), 4 replicates were run, identified as Aquarium A, Aquarium B, Aquarium C and Aquarium D.

3.2.2.1 Incubation of G. vermiculophylla

The experimental design was conducted considering four different salinities (16‰, 32‰, 48‰ and 64‰) and nine different times of incubation (1min, 5min, 10min, 30min, 1h, 3h, 6h, 12h and 24h), to analyse the combination of these conditions on the performance of the elemental composition levels on the *G. vermiculophylla* tissue.

Figure 3.6: *The four salinities 16‰, 32‰, 48‰ and 64‰ prepared for the first treatment.*

Figure 3.7: Thirty-six incubations for the first treatment.

It was made four different treatments essayed four different times in four consecutive days. Each treatment was based on thirty-six different incubations in total consisting of nine incubations for each salinity (16‰, 32‰, 48‰ and 64‰). Every incubation was performed using 500ml Erlenmeyer flasks containing 500ml of the artificial seawater with the respective salinity and 125µL of nutrient f/2 medium as represented in figure 3.7. Each treatment contained of approximately 4 g of *G. vermiculophylla* biomass, previously weighted before the treatment, and placed in a walk-in culture chamber, at a temperature of 16°C, with illumination of 90 µmol photons $m^{-1}s^{-1}$.

Each sample was left in a shaker for the specific period of time and salinity of the treatment, except for the control, which was subjected to the initial salinity of 32‰. After the specific time of each incubation, every sample was properly dried using a salad spinner, weighted, to obtain the final weight, and kept in labelled plastic bags to be frozen at -30°C (Fig. 3.8).

Figure 3.8: *Labelled samples at the end of each incubation.*

3.2.2.2 Preparing the different salinities.

The four saline solutions used for this experiment, were prepared following the Grashof simplified Artificial Sea Water protocol, diluting specific quantities of NaCl, Na_2SO_4, KCl, $MgCl_2$, $CaCl_2$ in distilled water to obtain the specific number of litters, with a specific salinity. In this experiment it was needed 20L of each saline solution for the four aquariums, so that the entire experiment could be carried out.

Table 3.1: Artificial Seawater calculations, made to obtain 1L of salinity 100‰, at the respective salinity wanted, based on the Grashof Number.

Source	**Grashof simplified**	
	basic	**I want**
quantity I want in lt	1	**1**
salinity I want	35	**100**
NaCl	24,99	**71,400**
Na2SO4	4,157	**11,877**
KCl	0,788	**2,251**
NaHCO3		
KBr		
H3BO3		
NaF		
MgCl2.6H20	11,13	**31,800**
CaCl2.2H2O	1,583	**4,523**
SrCl2.6H2O		
MgSO4.7H2O		

With that being said, it was concluded that preparing a certain amount of ASW 100‰ could be the best and most precise solution, and then dilute it in distilled water, until the desired salinity is reached.

In total, 32L of seawater 100‰ salinity was prepared according to the Grashoff Number (Table 3.1) and used 48L of distilled water. To reach the specific salinity needed, the calculations for the dilutions shown below were made considering that 5L of each salinity (16‰, 32‰, 48‰ and 64‰) was needed every day, for four days:

64‰ = $V_o*C_o = V_f*C_f \Leftrightarrow V_o = (5*64)/100 \Leftrightarrow V_o$ = 3.2L of ASW 100‰ in 1.8L of distilled water

48‰ = $V_o*C_o = V_f*C_f \Leftrightarrow V_o = (5*48)/100 \Leftrightarrow V_o$ = 2.4L of ASW 100‰ in 2.6L of distilled water

32‰ = $V_o*C_o = V_f*C_f \Leftrightarrow V_o = (5*32)/100 \Leftrightarrow V_o$ = 1.6L of ASW 100‰ in 3.4L of distilled water

16‰ = $V_o*C_o = V_f*C_f \Leftrightarrow V_o = (5*16)/100 \Leftrightarrow V_o$ = 0.8L of ASW 100‰ in 4.2L of distilled water

Salinity values were checked with a hand refractometer (Atago© S-20E, Japan).

3.2.2.3 Repeated Samples

In the Aquarium B experiment, some discrepancies were noted in the results when compared to the first Aquarium A experiment (Table 3.2), so those samples were measured again. The repeated samples were the ones considered for the results of the experiment (Table 3.3).

***Table 3.2*:** The first results of the respective samples.

Aquarium	Code	Salinity	Time (min.)	Start (hour:min.)	Finish (hour:min.)	Fwo (g)	Fwf	FWV (%)	DW	WC (%)	DW/FW
B	5	16	60	07:46	08:46	4.01	**4.12**	2.74	0.59	85.75	0.15
B	12	32	10	08:55	09:05	4.06	**4.13**	1.72	0.61	85.10	0.15
B	23	48	60	07:48	08:48	4.05	**3.57**	-11.85	0.57	83.88	0.14
C	24	48	180	07:42	10:42	4.04	**3.56**	-11.88	0.57	83.84	0.14

***Table 3.3*:** The new results of the respective samples, after being repeated.

Aquarium	Code	Salinity	Time (min.)	Start (hour:min.)	Finish (hour:min.)	Fwo (g)	Fwf	FWV (%)	DW	WC (%)	DW/FW
B	5	16	60	10:53	11:53	4.04	4.26	5.45	0.56	86.75	0.14
B	12	32	10	10:49	10:59	3.95	3.95	0.00	0.60	84.92	0.15
B	23	48	60	10:38	11:38	4.06	3.77	-7.14	0.63	83.22	0.16
C	24	48	180	13:12	16:12	4.05	3.62	-10.62	0.62	82.99	0.15

This difference in the results of experiment Aquarium B compared to the results of aquarium A may have been due to poor handling of the sample when removing it from the incubation or even when weighing it.

3.2.3 Fresh weight variation (%FWV)

Using the initial weight (FW_o) of the sample and the final weight (FW_f), it was calculated the seaweed fresh weight variation according to the sample's conditions. These results were measured from the GR (%) calculation, where the formula is shown below:

$$\mathbf{FWV\ (\%) = 100*(FW_f - FW_o)/FW_o}$$

3.2.4 Dry weight and determination of the water content

The frozen samples were removed from the freezer and immediately freeze-dried. The dry weight value was obtained by weighting the freeze-dried samples and the water content (WC) was measured using the FW_f and DW values, where the formula is shown below:

$$\mathbf{WC\ (\%) = ((FWf-DW)/FWf)\ *100}$$

3.2.5 Determination of elemental composition of the seaweed tissue

Approximately 0.5g of each freeze-dried sample was kept in a desiccator for elemental composition analysis. The elemental (As, Ca, Cd, Co, Cu, Cr, Fe, Hg, K, Mg, Na, Ni, Pb, Sn, Zn) determination was performed using Perkin Elmer Elan DRC-e Inductively Coupled Plasma-Mass Spectrometry, ICP-MS in a class 1000 clean room facility, at the Chemical Monitoring Facility, NUIG.

One-hundred fifty-six samples were analysed with 0,1g of sample, 3ml of nitric acid, 2ml of hydroxide peroxide, and 1ml of Milli-Q water in each tube, except for two tubes for the CRM (reference material) and one tube for the blank, where the blank was only nitric acid, hydrogen peroxide and Milli-Q. The two CRMs used were ERM-CD200 (Fucus) and GBW10023 (Laver). Unfortunately, a malfunctioning of the device made impossible to start the analysis of the samples.

3.2.6 Determination of tissue N and C content

Approximately 0.1 g of the freeze-dried sample was grinded for analysis of N and C content in the tissues. The samples were grinded using a TissueLyser II QIAGEN. In the case of nitrogen, the samples were sent to "Servizos de Apoio á Investigación" at the University of La Coruña (Spain), where tissue N contents was determined using a Flash combustion EA1108 elemental analyzer (Carlo Erba Instruments).

3.2.7 Statistical Analysis

Calculations and final graphical representations were performed using Microsoft Excel (2016). All data carried out in quadruplicate, from four experiments occurred in four different days (one per day) and presented as ± standard deviation of the mean (SD).

3.3 Results and Discussion

3.3.1 Fresh weight variation (%)

Analysing the initial results, the four experiments showed constancy in all assays (Fig. 3.9). It was observed that the samples incubated at lower salinity, showed positive values of their fresh weight variation compared to those at higher salinity, reaching up to 7.7% of FW at six hours (360 minutes) of incubation at salinity 16‰. It started with 4.02g of FW_o and ended with 4.33g of FW_f. On the other hand, samples subjected to salinity 64‰ had negative percentages of FWV, where the FW_f were lower than the FW_o, the lower value reaching -18.2% at the sample incubated for one hour (sixty minutes) from aquarium B experiment. The sample was 4.07g of FW_o and ended with 3.33g of FW_f.

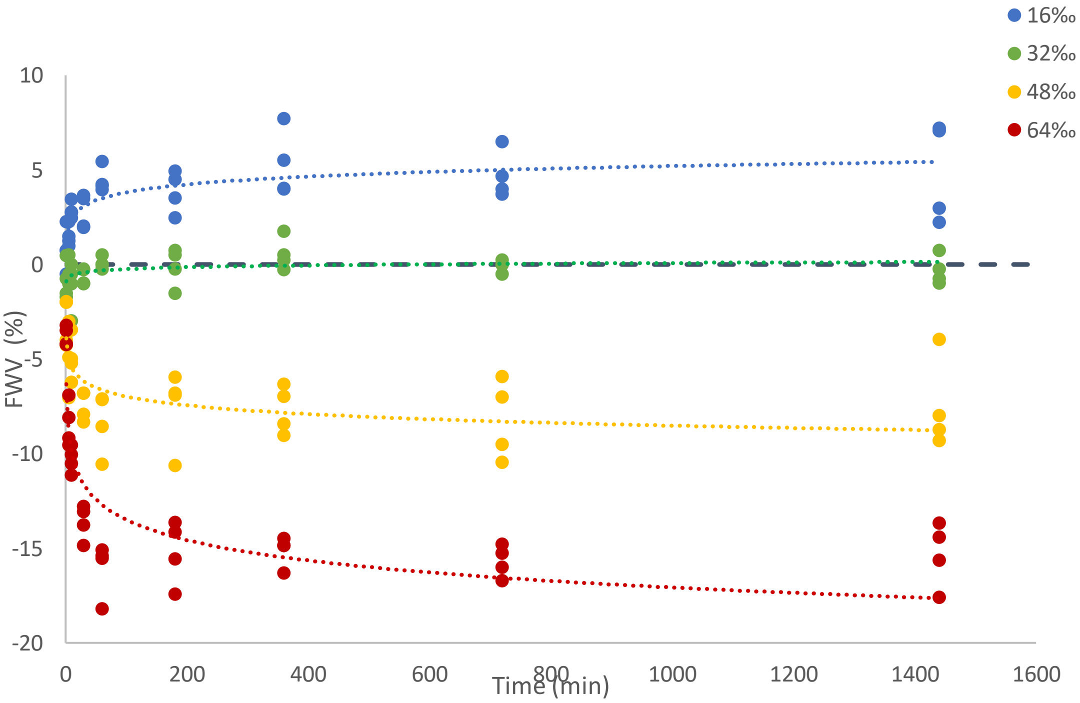

Figure 3.9: *Representation of the Fresh weight variation (%) of all samples of* G. vermiculophylla, *incubated in salinity 16‰ (blue dots), salinity 32‰ (green dots), salinity 48‰ (yellow dots), and salinity 64‰ (red dots), according to the incubation time (min). (n = 4)*

The effect of salinity on the fresh weight variation in seaweeds is a complex interaction influenced by various factors. According to **Sandoval-Gil et al., 2023**, elevated salinity levels create hyper-osmotic conditions, which may disturb the water balance between plant tissues and the surrounding seawater, resulting in cellular dehydration and dysfunction. This can result in a decrease of the fresh weight as the seaweed loses water. Additionally, higher salt concentrations induce hyper-ionic conditions, potentially causing ionic imbalances within the seaweed tissue **(Sandoval-Gil et al., 2023).** Consequently, this imbalance may lead to disruptions in cellular enzyme pathways and metabolic toxicity **(Bisson & Kirst, 1995).** Those scenarios are intricately linked to concepts termed as "limitation" and "disruptive stress" as outlined by **Davison & Pearson (1996)** in their study on seaweeds. These terms refer to the constrained water uptake and the physiological harm induced by excessive salinity, leading to ion-induced damage.

The samples incubated at 32‰ of salinity showed a constant variation, apart from the sample from the Aquarium D experiment, incubated for 10 minutes, where FWV levels were lower than normal, with a value of -2.97%. Samples exposed to 48‰ salinity had, as expected, lower values compared to those exposed to 32‰ salinity and higher than those exposed to 64‰ salinity.

The standard deviation was calculated from all the data that was obtained (Fig. 3.10). The sample with the highest standard error, was the sample incubated for 24 hours at 16‰ salinity, with a standard error of 4.87±2.28549.

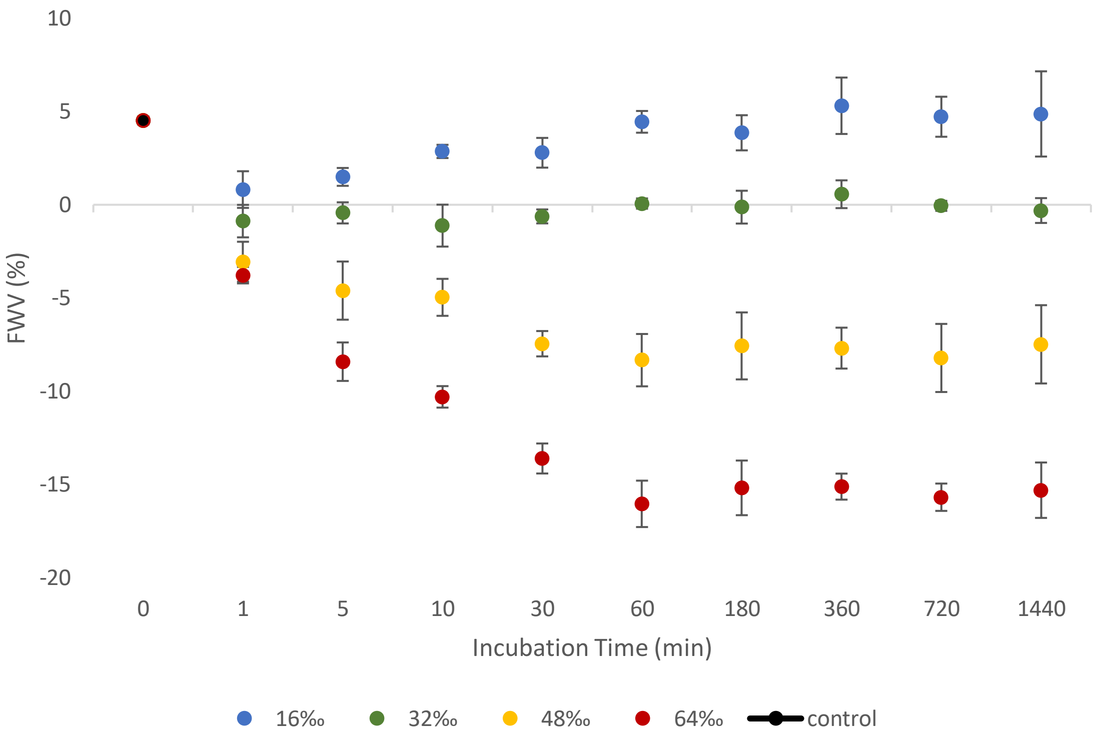

Figure 3.10: *Fresh weight variation (%) of all samples of* G. vermiculophylla*, incubated in salinity 16‰ (blue dots), salinity 32‰ (green dots), salinity 48‰ (yellow dots), and salinity 64‰ (red dots), and the control (black dot) according to the incubation time (min). The vertical bars at each point represent the value of the standard deviation of n = 4 samples.*

Based on the findings presented above, it was noted that regardless of the salinity level, there is a consistent duration for the cell to undergo the complete sequence of ecophysiological changes (i.e. process of the osmotic acclimation), until it reaches a state of stability. It can be noticed in all the samples that, during the initial sixty minutes of incubation, the cell undergoes changes and adapts osmotically. Following this period, the cell begins to stabilize.

One of the alternative explanations given by **Nejrup & Pedersen, 2012**, is that cell can have different types of regulation (i.e. regulation through ion exchange and changes in osmolytes). More studies have also shown that macroalgae firstly respond to changes in salinity by a rapid exchange of small ions that occur within minutes or a few hours **(Dickson et al., 1980; Reed, 1989; Nejrup & Pedersen, 2012)**. The degradation of organic osmolytes, on the other hand, occurs on a timescale of several hours to days, depending on the compound and species **(Kirst, 1990).**

That was confirmed by **Dickson et al., 1980,** in a study of *Ulva lactuta*, showing that the intracellular concentrations of small ions changed quickly with oscillations in salinity, while the amount of organic osmolytes remained almost unaffected at this timescale. Comparing that explanation with the results with *G. vermiculophylla*, that could be an alternative to explain the rapid variation during the first hour of salinity stress.

Estuarine species, with the capacity to cope with low and variable salinities, can tolerate greater variations in turgor pressure or cell volume than other species that inhabit environments with more stable and constant salinities **(Reed, 1983b)**, and this could be related to the increased elasticity of the cell wall, present in these species **(Kirst, 1990)**.

Subsequently, the reason why *G. vermiculophylla* can cope with low and variable salinity is still being investigated. It could be due to its fast and efficient osmoregulation, or simply because it tolerates changes in turgor pressure or cell volume **(Nejrup & Pedersen, 2012)**.

3.3.2 Water content (%)

Previously, it was concluded that changes in salinity are followed by a flux of water along an osmotic gradient, which can affect turgor pressure and/or the volume of the cell **(Nejrup & Pedersen, 2012).** By measuring the water content of the samples, it was possible to determine the actual amount of plant material present, which is crucial for an accurate assessment of biomass and growth rate.

Once again, we can observe in Figure 3.11, that samples subjected to lower salinity showed higher results. This may have occurred because, as ASW 16‰ contains fewer salts, and the cells eventually end up absorbing more water during the osmotic acclimation process. The algae incubated at 16‰ salinity for 12 hours, had the highest water content, at 89.62%.

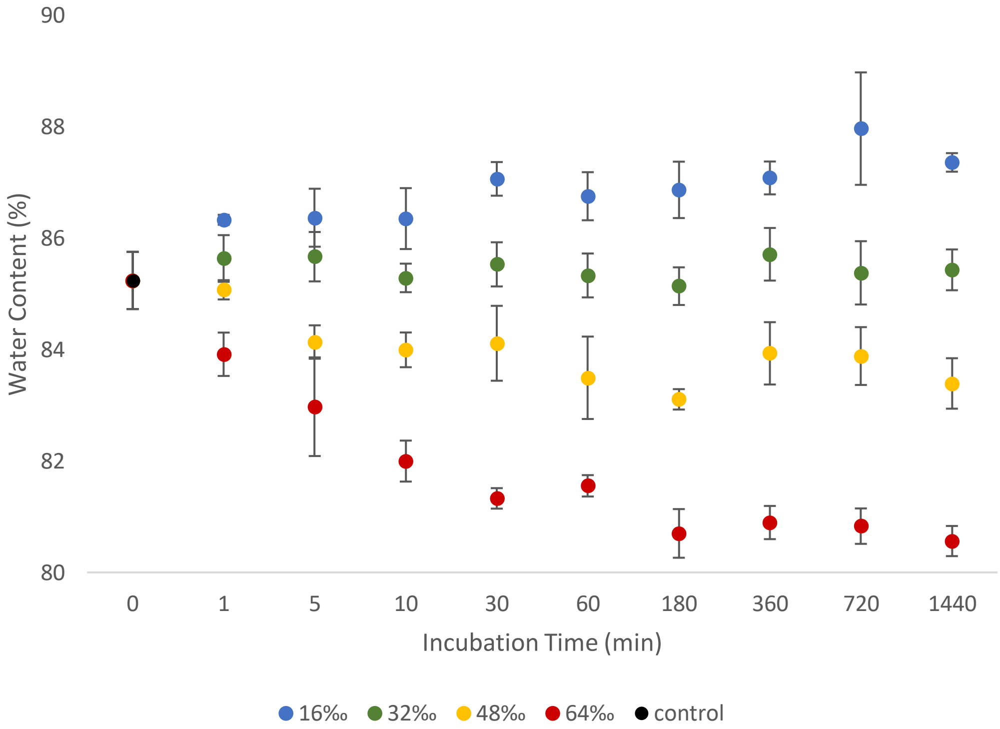

Figure 3.11: *Water Content (%) of all samples of* G. vermiculophylla*, incubated in salinity 16‰ (blue dots), salinity 32‰ (green dots), salinity 48‰ (yellow dots), and salinity 64‰ (red dots), and the control (black dot) according to the incubation time (min). The vertical bars at each point represent the value of the standard deviation of n = 4 samples.*

According to **Karsten, 2012**, when the intracellular water potential is lower than that of the external medium, there is a water influx, which means that water is taken up in all living cells by osmosis, driven by the water potential gradient. Therefore, seaweeds create an internal osmotic potential higher than that of seawater to gain and retain constant water content of the cells, which is necessary to maintain turgor as the driving force for growth **(Kirst, 1990; Karsten, 2012)**. That explains the reason why the samples incubated to lower salinity showed a higher water content.

Due to the higher salt content in ASW 64‰, samples incubated at this salinity showed lower water content (WC, %) values. This is because there is less water available to be absorbed and more salts present. The sample with the lowest WC percentage, with a value of 80.16%, was obtained after three hours of incubation.

As it was previously observed in the GR (%) responses, it takes also approximately sixty to one-hundred and eighty minutes for *G. vermiculophylla* cells to stabilize their absorption. This was explained in a previous study, which found that the water fluxes during the phase I of the osmotic acclimation are rapid processes with half times in seaweeds lasting from minutes to hours **(Zimmermann & Steudle, 1978)**. Under hyposaline (lower salinity) conditions, there is an influx of water, while during hypersaline (higher salinity) stress, there is an efflux of water. Both processes help to moderate osmotic stress, at least temporarily **(Karsten, 2012).**

3.3.3 Percentages of N and C

3.3.3.1 Nitrogen (%)

According to **Hanisak, 1983**, nitrogen is the nutrient most frequently cited as limiting the growth of seaweeds in natural environments. Seaweeds have physiological mechanisms to acquire, utilize, and store various forms of nitrogen in environments under tremendous spatial and temporal variations in nitrogen concentration **(Hanisak, 1990)**.

However, the osmotic pressure, which is influenced by the salinity concentration, affects the moisture distribution inside and outside the semipermeable membrane and the absorption of nutrients by seaweed **(Ding *et al.*, 2013)**. The tissue nitrogen in *Gracilaria vermiculophylla* differed evidently between different incubation times (see Figure 3.12). of the percentage of biomass increased to 3.58% after ten minutes of incubation with a salinity of 32‰ and decreased to 2.68% after thirty minutes of incubation with a salinity of 64‰. In another study with *Ulva pertusa*, the relationship between salinity and nitrogen content was analysed, and it was found that nitrogen levels significantly decreased after an exposure to a salinity of 40‰ **(Choi *et al.*, 2010).**

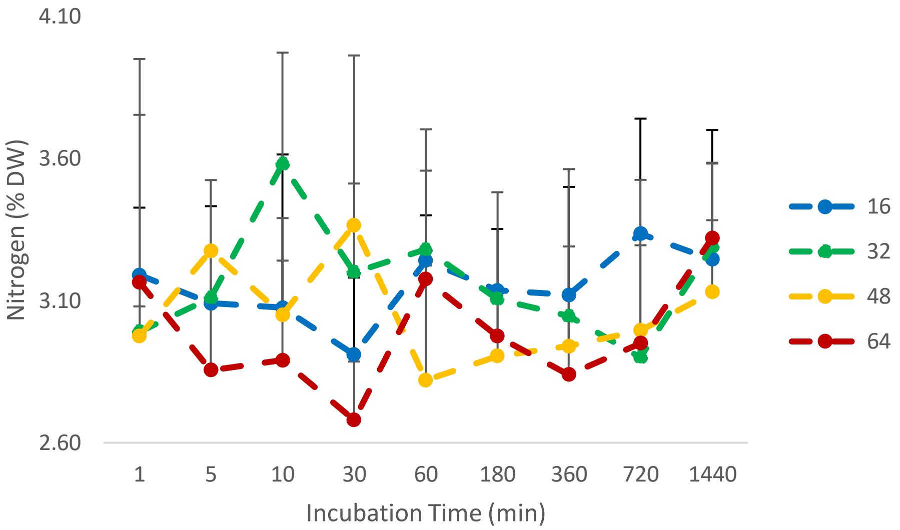

Figure 3.12: *Percentage of Nitrogen of all samples of* G. vermiculophylla*, incubated in salinity 16‰ (blue dots), salinity 32‰ (green dots), salinity 48‰ (yellow dots), and salinity 64‰ (red dots) according to the incubation time (min). The vertical bars at each point represent the value of the standard deviation of n = 4 samples.*

That could be explained because changes in salinity can directly affect osmoregulatory processes, which may impact the overall physiological conditions of seaweeds when submitted to high salinity values, including their ability to take up and utilize nitrogen **(Smyth & Elliott, 2016)**. That could explain why the lower percentages of nitrogen analysed were present in samples exposed to a salinity of 64‰ (except with the sample incubated in ASW 48‰, for one hour, that the value was lower than the sample at 64‰).

Li *et al.*, 2019, also analysed the effect of salinity on the nitrogen content of *Sargassum fusiforme* and found that the responses were like those observed in *Gracilaria vermiculophylla*, showing that the percentage of N was mostly close to 3% when the species were submitted to salinities of approximately 20‰ and 30‰.

3.3.3.2 Carbon (%)

Carbon is essential for the growth, structure, energy dynamics, and metabolic processes of seaweed **(Gao et al., 2022)**. The seaweed's ability to fix carbon through the photosynthesis not only sustains their own growth and survival, but also has broader ecological implications, influencing marine ecosystems and contributing to the global carbon cycle **(Gao et al., 2022)**.

Analysing the results of *Gracilaria vermiculophylla* it was shown that the percentage of carbon did not change significantly between incubation times, except for the sample incubated for sixty minutes at a salinity of 64‰, which yielded the lowest value of 27.26% (see Figure 3.13). Thus, the samples subjected to higher salt concentrations exhibited a significant decrease in the first hour of incubation, followed by an increase thereafter. According to **Macler, 1988**, in a study on the effect of salinity on photosynthesis, carbon allocation and nitrogen assimilation in the red algae, *Gelidium coulteri*, it was observed that the carbon fixation decreased when the algae was submitted to salinities higher than normal, in this case, salinities ranging from 10 to 50‰.

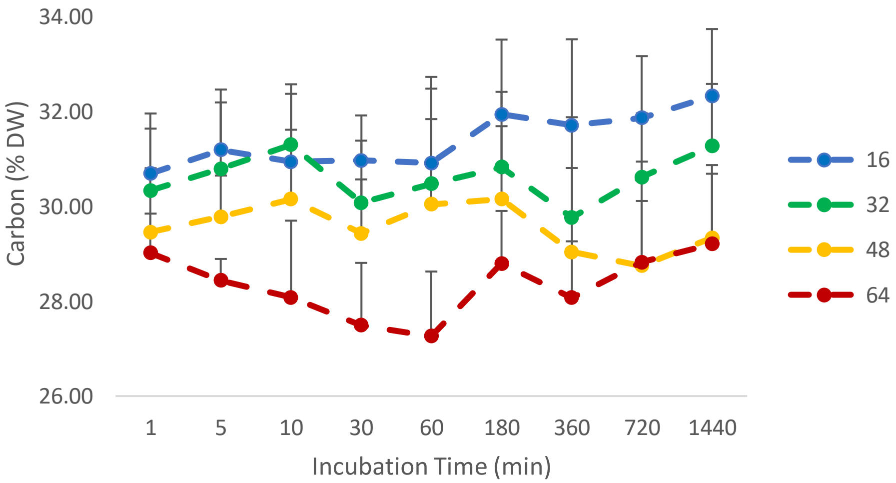

Figure 3.13: *Percentage of Carbon of all samples of* G. vermiculophylla*, incubated in salinity 16‰ (blue dots), salinity 32‰ (green dots), salinity 48‰ (yellow dots), and salinity 64‰ (red dots) according to the incubation time (min). The vertical bars at each point represent the value of the standard deviation of n = 4 samples.*

The highest values recorded for *G. vermiculophylla* were observed at a lower salinity, 16‰, at 1440 minutes of incubation, with a value of 32.33%. Comparing to the study made by **Li et al., 2019**, on the effect of salinity and temperature on carbon levels of *Sargassum fusiforme*, it was shown that under a temperature of 15º C, similar to the temperature used for the experiment with *G. vermiculophylla*, 16ºC, the carbon content of seaweed incubated at salinities between 10 and 20‰, was significantly higher than that at 40‰. Analysing these responses, it is evident that salinity stress has a negative impact on enzymatic activities associated with carbon fixation, ultimately affecting growth, as shown by the GR% results. Plus, salinity stress may decrease carbon percentages by accelerating the respiratory activity **(Li et al., 2019)**.

Analysing the difference between the results of N and C content it is inevitable to notice the discrepancy between the two outcomes. For instance, a significant decrease in carbon percentages was observed in the samples incubated for sixty minutes with a salinity of 64‰. On the other hand, it occurs a notable increase in nitrogen levels on the same samples, suggesting that nitrogen levels influenced carbon levels. That could happen when the macroalgae is submitted to specific conditions, suggesting that their variations occur from a same cause. **Lee & Dunton (1999)** observed that sediment nitrogen availability influences the C and N dynamics in seagrass, with higher tissue N content and C content under higher sediment nitrogen conditions. The N and C content in seaweeds are influenced by a variety of factors, including ambient nitrate concentrations **(Young et al., 2007)**, and sediment nitrogen availability **(Lee & Dunton, 1999)**. These studies collectively suggest that the N and C content in seaweeds are interlinked and influenced by environmental factors, and sometimes influenced by the same conditions, equally or differently.

A related situation was also observed in one study from **García-Sánchez et al., 1993**, with *Gracilaria tenuistipitata*, reporting that tissue carbon content decrease in response to nitrogen assimilation. It was found that these conclusions could be correlated to the potential rivalry for reducing power and ATP between nitrogen and carbon metabolic rate in these species **(Turpin, 1991)**.

3.4 Conclusion and Future Prospects

The findings of this study provide valuable insights into the effect of the salinity stress on the growth and biochemical composition of *Gracilaria vermiculophylla*. Through a series of short incubation protocols, it was observed that increasing the salinity levels had a significant effect on growth and water content, responding to the osmotic acclimation of the cells. High salinities, above 40%, were mainly associated with reduced growth rate, water content, percentages of carbon and hydrogen, except for nitrogen where no differences were observed.

On the other hand, it was also noticed that the responses of this species to lower salinities, mostly between 16-30%, were very much optimistic, showing higher growth percentages, high levels of water contents, and levels of carbon and hydrogen contents.

These results are largely consistent with those found in other studies and scientific articles on the subject. Unfortunately, the limited resources available to carry out this experimental test led to a number of shortcomings that prevented me from collecting all the data necessary to complete this study. Specifically, in this experimental test, the collection of samples was conditioned by the impossibility of analysing the elemental composition of the seaweed tissue, which was one of the most crucial parts of this study.

Looking ahead, there are several promising avenues for future research in this area. Firstly, further investigation is warranted to explore the analysis of the elemental composition of the *G. vermiculophylla* tissue submitted to these short incubation protocols. Therefore, the comparison of the levels of the desirable elements (K, P, N), in order to optimize this large biomass generated by the macroalgal blooms, improving their composition, in which, in future studies, these seaweeds could be used for human applications, possibly agriculture with the production of biofertilizers.

4. Internship Report Conclusion

The opportunity to undertake an internship in a research group environment provides the intern to undertake several professional and personal challenges that are difficult to achieve during in an academic environment. The internship experience with the MACROMAN Project has provided valuable insights into the practical application of biotechnology in the field of marine resources.

Through hands-on involvement in various projects and tasks, the intern was able to develop a deeper understanding of macroalgal blooms and macroalgae in general, including their physiology, cultivation, and maintenance. It also provided a deeper knowledge of working as a professional in a laboratory environment, giving more confidence and experience when entering the job market.

The MACROMAN Project based in the Ryan Institute at the National University of Ireland, offers a platform to apply the academic knowledge acquire during the internship path at the Polytechnic University of Leiria, in diverse settings and to acquire new professional skills. Additionally, it also fosters personal growth by developing a sense of responsibility, organizational skills, the ability to communicate in a different language, and the ability to build relationships. Moreover, the MACROMAN group provides excellent facilities and equipment, facilitating the high-quality work and equipping interns with essential tools for the professional development.

The final goal for this internship was to develop a short incubation protocol to improve of the elemental composition of the macroalgal species *Gracilaria vermiculophylla*, taking advantage of the rapid ecophysiological response to four different salinity ranges and nine incubation times.

In conclusion, all the work carried out during the six months internship proved to be a great opportunity to enter in the job market. The research group recognized the work done by offering a position after the internship.

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