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Aplicação de extratos das macroalgas Ulva sp. e Chondrus crispus como biofertilizantes em Couve e Aveia

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ana Marta Gonçalves, Investigadora do Centro de Ciências do Mar e do Ambiente, Departamento de Ciências da Vida da Universidade de Coimbra e do Departamento de Biologia, Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e da Doutora Cláudia Sofia Cordeiro Nunes, Investigadora do Departamento de Engenharia de Materiais e Cerâmica da Universidade de Aveiro.

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palavras-chave Macroalgas, Agricultura, Fertilizantes, Bioestimulantes, Sustentabilidade, Ulvana, Carragenana, Germinação.

resumo

A agricultura é uma das principais fontes de emissões de gases de efeito estufa e perturbação ambiental. Com a expectativa de crescimento da população humana nas próximas décadas, novas formas de aumentar a produção agrícola de forma sustentável e minimizar poluição são necessárias. Neste contexto, esta investigação explora o potencial das macroalgas, especificamente *Ulva* sp. e *Chondrus crispus* da costa portuguesa, como biofertilizantes para duas culturas agrícolas estrategicamente importantes, couve e aveia.

A extração com água quente foi avaliada como um método de produção de extratos de macroalgas com capacidades biofertilizantes. O estudo envolveu uma caracterização física e bioquímica abrangente dos extratos de macroalgas, que revelaram condutividade elétrica e viscosidade distintas e adequadas para serem usadas na agricultura. Os extratos obtidos são ricos em macro e micronutrientes essenciais para o desenvolvimento das plantas, como os polissacarídeos e compostos fenólicos com atividade bioestimulante.

O extrato de *Ulva* sp. melhora significativamente a germinação de plântulas de aveia em termos de tamanho, peso e conteúdo de clorofila, enquanto o extrato de *Chondrus crispus* produz resultados menos favoráveis. No caso da germinação de sementes de couve, ambos os extratos melhoram o vigor das plântulas, sendo que o extrato de *Chondrus crispus* exibe os efeitos mais promissores no tamanho, peso e concentração de pigmentos das plântulas. Por outro lado, a aplicação foliar dos extratos em plantas jovens de aveia a crescer em condições ideais não produziu diferenças observáveis.

Este trabalho confirmou o potencial dos extratos de macroalgas para melhorar o vigor das plântulas, oferecendo perspetivas de aumento na produção agrícola. Este estudo permite contribuir para uma agricultura sustentável e para a preservação ambiental.

keywords Macroalgae, Agriculture, Fertilizers, Biostimulants, Sustainability, Ulvan, Carrageenan, Germination.

abstract

Agriculture is a major source of greenhouse gas emissions and environmental disruption. With the human population expected to grow in the coming decades, new and sustainable ways of increasing agricultural production and minimizing pollution are needed. To address this objective, this research explores the potential of macroalgae, specifically *Ulva* sp. and *Chondrus crispus* from the Portuguese coast, as biofertilizers for two strategically important crops, cabbage and oats.

Hot water extraction was evaluated as a method of producing macroalgae extracts with biofertilizer capabilities. The study involved a comprehensive physical and biochemical characterization of the macroalgae extracts, revealing distinctive and adequate electrical conductivity and viscosity. These extracts are rich in macro and micronutrients essential for plant growth, such as polysaccharides and phenolic compounds with biostimulant activity.

Ulva sp. extract significantly enhances oat seedling germination in terms of size, weight, and chlorophyll content, whereas *Chondrus crispus* extract yields less favorable outcomes. In the case of cabbage seed germination, both extracts improve seedling vigour, with *Chondrus crispus* extract exhibiting the most promising effects on seedling size, weight, and pigment concentration. In contrast, the foliar application of the extracts in oat juveniles growing in ideal conditions did not produce observable differences.

This research confirmed the potential of macroalgae extracts to enhance seedling vigor, offering prospects for improved agricultural yields. The findings of this study could contribute for sustainable agriculture and environmental preservation.

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List of Abbreviations

FAO	Food and Agriculture Organization of the United Nations
EC	Electrical Conductivity
MMB	Borane 4-methylmorpholine
TFA	Trifluoroacetic acid
GalA	Galacturonic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
FAME	Fatty Acid Methyl Ester
UE	*Ulva* sp. extract
CE	*Chondrus crispus* extract

1. Introduction

1.1. Trends in the agricultural market

Agriculture has played a pivotal role in the development of human civilization, facilitating the transition to permanent settlements, allowing society to flourish, and driving technological progress.[1].

According to the Food and Agriculture Organization of the United Nations (FAO), primary crop production grew by 54% between 2000 and 2021 while population growth was 29%[2]. Despite this growth in crop production, the FAO reported in 2022 that 735 million people faced hunger, and 2.4 billion people experienced food insecurity worldwide[3]. Projections by the United Nations indicate that the global population will continue to grow, reaching 9.7 billion by 2050 and peaking at nearly 10.4 billion in the mid-2080s[4].

However, this impressive upscaling of agricultural output has come at a great cost. The main cause of deforestation is the creation of new land for agriculture[5]. Intensive agriculture practices harm the soil in various ways, they disrupt the activity of microorganisms in the soil and lead to the loss of organic matter and essential nutrients. These adverse effects reduce agricultural productivity[6]. Additionally, the of leaching agricultural inputs into surface waters and groundwaters[7], disrupts ecosystems and creates sanitary risks[8].

The global food system is estimated to account for a significant portion of annual emissions, ranging from 21% to 37%[9]. In particular, agriculture is the largest anthropogenic source of N_2O, considered the primary ozone-depleting substance as well as a greenhouse gas approximately 265 more powerful than carbon dioxide (CO_2)[8].

Industrial agriculture faces additional challenges, including heavy reliance on non-renewable inputs such as energy for mechanical operations, fertilizers, and pesticides, which may be depleted in the coming decades if current trends persist[10].

In today's globalized world, events in one region can disrupt the delicate balance of the global agricultural market, leaving import-dependent countries vulnerable to supply shocks and food inflation. For instance, in Portugal, where cereal production has declined 37% in

recent decades[11], cereal import dependence leaves the country more vulnerable to supply shocks and food inflation[12].

Moreover, geopolitical conflicts, such as that involving, Russia, Belarus, and Ukraine who are major players in the agricultural market, both in the production of the main agricultural inputs[13], as well as the grain market, can have profound effects. Between September 2021 and September 2022, nitrogen fertilizer prices in the EU rose by 149%, while mined fertilizer prices doubled. Figure 1 illustrates the price movements, although fertilizer prices experienced a substantial decline since October 2022, they remain elevated when compared to previous years[14].

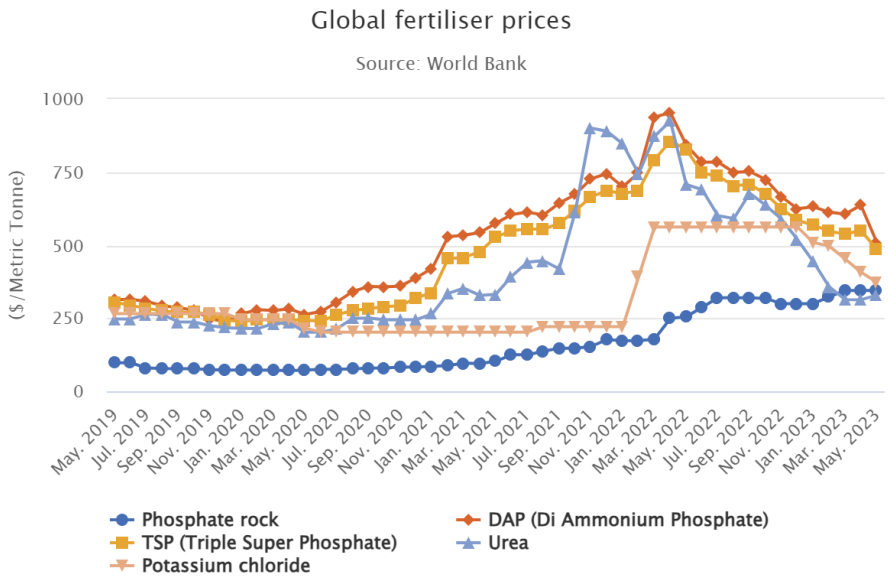

Figure 1- Evolution of fertilizer prices since May 2019

Source: World Bank

Russia and Ukraine accounted for 28% of the world's total wheat exports in 2020, following the Ukraine invasion, diminished fertilizer, and cereal exports caused the Food Price Index to reach a record high, increasing by 12.6%[15]. Before the conflict, Ukraine, followed by Spain, France, and Brazil, was one of the primary grain sources for the Portuguese market[11].

Similarly, crop yields are dependent on climate factors. Climate change is increasing the frequency and severity of heatwaves, droughts, and floods, all of which have the potential to disrupt the agricultural market[16]. In response to the forecasted 2024 El Niño Southern Oscillation, a natural phenomenon that impacts weather patterns[17], India banned rice

exports to control domestic food prices although increasing food inflation in other Asian countries[18].

Concerns for climate change, environmental health, and food security are growing amid these challenges. Resources are now being dedicated to researching alternative methods that reduce dependence on large supply chains, minimize resource consumption, and stabilize markets.

A report by FAO highlights how Agroecology, a set of practices aimed at ensuring sufficient, safe, and nutritious food while respecting human rights, developed for the 2030 Agenda for Sustainable Development[19], contributed to shielding communities from agricultural market disruptions in recent years. By promoting practices such as diversification, crop-livestock integration, intercropping, and waste recycling, Agroecology contributes to nutrient cycling, biodiversity conservation, pollution reduction, and the provision of affordable and nutritious food for communities[20].

Agroecology thus relies on natural resources to enhance soil fertility and improve crop yields. These organic materials typically include plant by-products, animal manure, and leftover organic waste. This distinction allows for local sourcing, thereby minimizing the need for transportation and reducing reliance on external suppliers[20]. Furthermore, organic fertilizers release nutrients slowly, in contrast to the rapid nutrient release of inorganic fertilizers. This gradual nutrient release provides long-term soil protection, enhancing its physical characteristics and aeration while ensuring nutrient availability[21].

1.2. Opportunities in the Ocean

The ocean presents a compelling alternative source of fertility due to its vast size and continuous nutrient enrichment from various sources, including atmospheric deposition, river runoff, and inputs such as fertilizers, sewage, and industrial runoff, particularly in coastal areas [22]. Approximately half of the planet's net primary production is attributed to the ocean, with coastal areas playing a pivotal role in the total net primary production of the

ocean [23]. These areas are predominantly inhabited by macroalgae due to their requirement for adequate light and an attachment point. These organisms exhibit high carbon fixation capacity, providing support for trophic webs and regulating nutrient levels[24].

1.2.1. Macroalgae as an environmentally sustainable resource

Macroalgae, characterized by their diversity and spectrum of colors, can thrive both at the sea surface and affixed to substrates several meters below. These resilient organisms endure rapid fluctuations in temperature, salinity, and occasional exposure to air during tidal changes[25]. This resilience is partly attributed to their prolific production of biologically active compounds, contributing to their adaptability[26]. Macroalgae have numerous potential applications, a few of macroalgae biologically active compounds applications are summarized in Figure 2.

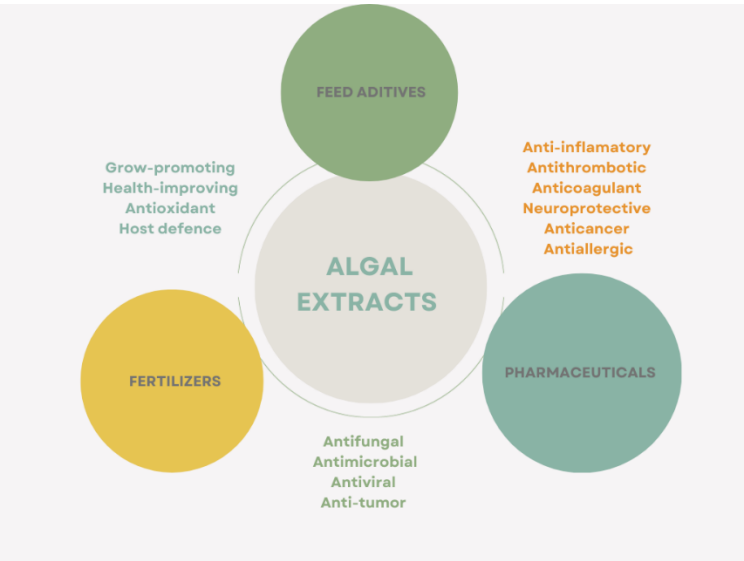

Figure 2- Potential applications of macroalgae bioactive properties.

Adapted from Chojnacka, et al. (2012) [27].

Humans have harnessed the versatility of macroalgae for millennia. Initially used for food, feed, and traditional medicine, macroalgae found their place in various industries, such as the manufacture of gels, cosmetics, and pharmaceuticals[28]. Coastal communities worldwide, including those in Portugal, have long recognized the value of macroalgae as a

nutrient-rich organic material, utilizing it as both a soil conditioner and fertilizer[29]. Macroalgae can contribute to the renovation and adaptation of agricultural practices in the areas of soil, water, heat, disease, and nutritional management[30].

When assessing potential alternative sources of fertility, cost and availability are critical considerations, particularly for farmers operating on narrow profit margins. Macroalgae represent an abundant and renewable resource, with macroalgae estimated to produce approximately 1.32 x 10^{12} kg/year, covering extensive areas ranging from 6.06 to 7.22 million km^2[31]. Furthermore, the frequency of algal blooms is increasing due to factors like eutrophication and climate change[32]. This phenomenon has led to a growing concern over Marine Macroalgae Waste, as these blooms result in substantial amounts of decomposing biomass, adversely affecting biodiversity, human health, and the recreational value of coastal areas. Conventional methods for managing Marine Macroalgae Waste often incur significant environmental and economic costs[33]. Hence, a compelling environmental, social, and economic incentive exists to repurpose Marine Macroalgae Waste into value-added products, notably fertilizers [34].

Algae fertilizers, derived from algal biomass, offer versatile applications in agriculture, serving as biofertilizers, biopesticides, biostimulants, and soil stabilizers [35]. These solutions can be strategically applied to various parts of plants, including leaves and seeds [36], or integrated into the soil as amendments, providing a gradual nutrient release over time [37].

1.2.2. Macroalgae as fertilizers

One of the remarkable features of macroalgae is their transformative impact on soil properties, encompassing physical, chemical, and biological aspects. Their application not only enhances agricultural productivity but also curtails nitrogen and phosphorus leaching compared to traditional livestock manures. Consequently, it contributes to improve the quality of water draining from the soil[35].

Within the mineral composition of macroalgae, prominent microelements often include potassium, sodium, magnesium, and calcium. Additional microelements, such as copper, iron, manganese, and zinc, are also found in varying quantities [38].

As a result of macroalgae growing medium and high mineral content, it is advisable to wash macroalgae before application to mitigate the potential negative effects of salinity on plant germination and growth. Elevated osmotic pressure resulting from salinity may impact on nutrient uptake[39]. Thus, careful control of the electrical conductivity (EC) of macroalgae compost and fertilized soil becomes paramount.

The protein content in marine algae varies among species. Generally, brown macroalgae exhibit low protein levels, ranging from 3% to 15% on a dry weight basis (DW). Distinctively, green macroalgae typically fall within the moderate range of 9% to 26% DW, while red macroalgae can reach an impressively high protein content of 47% DW [40].

Conversely, lipid content in macroalgae species tends to be relatively low, often less than 5% on a dry weight basis. Variations in lipid quantity and fatty acid profiles can be attributed to environmental factors, as well as genetic differences among species[41].

Macroalgae are rich repositories of diverse molecules, including polysaccharides, which constitute an average of 50% of the algae's composition. The total carbohydrate concentrations in macroalgae species can account for up to 76% of their dry weight[42]. These polysaccharides include alginate, agar, carrageenan, fucoidan, laminaran, and ulvan and have been shown to enhance stress tolerance and promote growth in plants[43]. Alginate is a polysaccharide found in brown algae. It has been shown to enhance soil structure, provide an organic matter source, and improve soil aeration and capillary activity. Moreover, alginates bolster water retention capacity, effectively reducing erosion, promoting plant root growth, and enhancing microbial activity[44][44]. Remarkably, alginate can be harnessed for the engineering of biodegradable, slow-release pesticides or fertilizers[45].

The use of macroalgae biomass can be very beneficial when used near the sources of this resource, however, at higher transport distances, economic and environmental costs increase rendering this solution unattractive.

1.2.3. Macroalgae as Biostimulants

Figure 3 represents the typical value pyramid of biomass, at the base are applications with low value per volume ratio, at the top applications with high value per volume ratio, typically

low-value applications are used in proximity to the biomass source while in high-value applications transport is a lower percentage of the overall cost. Macroalgae extracts provide higher economic and environmental efficiency as they typically have higher concentrations of the most bioactive macroalgae substances, the resulting waste of these extractions can be used in lower-value products such as biofuels[46].

Figure 3- Macroalgae biomass value pyramid

Adapted from: Bennett et al. (2023) [46]

These extracts find extensive application as plant biostimulants, categorically described as substances or microorganisms applied to plants to improve diverse attributes, such as nutritional efficiency, resilience to environmental stresses, and overall crop quality characteristics[47]. Despite often including relevant amounts of essential nutrients, namely minerals[48], macroalgae extracts are mainly classified as biostimulants rather than fertilizers, representing a substantial part of the growing biostimulant market[46].

Numerous reviews have been conducted on the biostimulatory effects of macroalgae extracts, the benefits include increased stress tolerance, growth promotion, enhanced nutrient absorption, and increased crop productivity [49]. Furthermore, they play a role in breaking dormancy, nurturing root system growth, enhancing the functions of photosynthetic and other vegetative tissues, boosting plant vigor and uniformity, controlling flowering patterns, inducing fruit formation and maturation, and extending produce shelf life[50–52].

Macroalgae extracts are complex solutions, often composed of multiple bioactive substances, including polysaccharides, vitamins, antioxidants, pigments, and hormones[53]. In certain studies, researchers evaluated the use of purified components of these extracts. Tobacco plants treated with three different oligo-carrageenans showed increased leaf biomass but also improved various metabolic processes, specifically, they promoted photosynthesis, enhanced the efficiency of photosystem II, increased chlorophyll levels, and promoted the activity of the ribulose 1,5-biphosphate carboxylase/oxygenase (rubisco) enzyme[54]. In a study evaluating the biostimulant activity of three brown macroalgae polysaccharides in high salinity conditions, researchers found that all promoted plant growth, reduced membrane lipid peroxidation, increased chlorophyll content, improved antioxidant activities, and helped to regulate intracellular ion levels. Furthermore, the results suggested that molecular weight and sulfate content of the polysaccharide play a role in saline tolerance[43].

Additionally, macroalgae produce a wide range of phytohormones, similar to those found in land plants[55]. Phytohormones like auxins, gibberellins, cytokinins, abscisic acid, ethylene and brassinosteroids play critical roles in regulating plant growth, development, and responses to environmental stimuli. Phytohormones can enhance plant metabolism and enzyme activity, leading to improved crop yields and quality[56]. They can be extracted from macroalgae and directly applied as biostimulant extracts, or they can be integrated into organic fertilizers made from plant materials or microorganisms[57].

While employing isolated macroalgae compounds can be effective, there is compelling evidence to suggest that utilizing the entire extract is optimal, as its components exhibit synergistic interactions[58]. Although the underlying metabolic mechanisms responsible for macroalgae extracts' effectiveness are still uncertain[53].

An *Ascophyllum nodosum* extract applied in rapeseed increased chloroplast quantity by regulating MinE gene expression[59]. In a subsequent study, the same extract was shown to enhance the translocation of iron (Fe) and zinc (Zn) from the roots to the shoots, stimulate root growth and the uptake of macronutrients like nitrogen (N), sulfur (S), potassium (K), and phosphorus (P). Additionally, the extract application led to increased plant concentrations of magnesium (Mg), manganese (Mn), sodium (Na), and copper (Cu). These effects are associated with the upregulation of specific genes involved in nutrient transport, such as a copper transporter (COPT2) and NRAMP3, a gene potentially linked to the translocation of iron and zinc[60].

Another study explored *Ascophyllum nodosum* extract's impact on drought-stressed soybean plants. The treatment enhanced resilience, reducing wilting and oxidative damage while promoting ABA production and photo tolerance, by regulating different gene pathways. Additionally, Aquaporin gene GmPIP1b expression was related to improved water movement, genes GST and GmBIP with decreased oxidative damage, and GmTP55 with reduced lipid peroxidation-derived aldehydes[61].

On a similar note, *Ulva* sp. extract applied to a model legume protected the plant against a pathogenic fungus by inducing the expression of various defense-related genes in the plant, including genes associated with phytoalexin and phenylpropanoid biosynthesis, as well as several pathogenesis-related (PR) genes. Notably, primary metabolism genes related to carbon and nitrogen metabolism were not downregulated[62]. The increased biotic stress tolerance provided by *Ulva lactuca* seems to be related to both high concentration of phenolic compounds[63], and, its characteristic sulfated polysaccharide, ulvan[64].

An interesting study compared commercial macroalgae extracts to a standard cytokinin called trans-zeatin riboside (t-ZR), in heat stress conditions. The results showed that both the macroalgae extracts and t-ZR treatments increased the levels of t-ZR in the grass leaves and improved various aspects of plant performance. They increased superoxide dismutase and nitrate reductase activity and slowed the decline caused by heat stress in grass quality, photochemical efficiency, and root viability. Notably, the mineral component of the macroalgae extract did not seem to confer these advantages[65][66], indicating that the effects of macroalgae extract are more oriented toward biostimulation rather than fertilization.

1.3. Crops of interest: *Brassica oleracea* and *Avena sativa*

Brassica vegetables are low in fat, but high in vitamins, minerals, and fiber. Their various phytochemicals prevent oxidative stress, stimulate the immune system, and reduce cancer risk[67].

Cabbage production requires high amounts of nutrients, usually provided by synthetic fertilizers[68]. On the same note, cabbage cultivation can be adversely affected by pests and disease. Downy mildew, cabbage aphid, and terrestrial gastropods are commonly controlled with synthetical pesticides, adversely affecting environmental and human health[69–71].

Studying the effectiveness of biostimulants in mitigating the challenges of cabbage cultivation can yield significant benefits, especially considering that in 2021, global cabbage production reached 71 million tons, solidifying its position as one of the world's most widely consumed vegetables[72].

As anticipated from studies in other plants, cabbage plants treated with Eckol, a phlorotannin compound from brown macroalgae, showed increased growth and resistance against cabbage aphid infection[71].

Avena sativa has been one of the main cereal crops, through the years used as food and feed[73]. Recently, oats consumption has increased because of the rise in veganism[74], and its use as a therapeutic agent due to their beneficial effects on lipid regulation and cognitive function[75,76]

Historically, Europe has been the main producer of oats[72], unsurprisingly, the geopolitical conflict in Eastern Europe has caused a sharp rise in the price of oats in consumer markets[77].

There is a high incentive in Portugal to increase *Avena sativa* production to reduce prices to consumers and decrease import dependence. Drought and extreme temperatures severely impact oat cultivation yield[74], these phenomena are increasing in frequency, therefore, sustainable ways of dealing with these challenges are needed.

1.4. *Ulva* sp. and *Chondrus crispus* extracts as potential agricultural tools

Ulva sp. and *Chondrus crispus* (Figure 4) are commonly found in Portuguese shores. Their abundance is relatively stable throughout the year[78], which is important for continuous industrial production.

Figure 4- Ulva sp. *(left) and* Chondrus crispus *(right)*

Ulva sp. is a fast-growth, high photosynthetic efficiency macroalgae[79]. This genus is a significant source of marine macroalgae waste due to eutrophication[80]. *Ulva* sp. belongs to the class Ulvophyceae within the phylum Chlorophyta. The green color of this algae primarily results from the proportion of chlorophyll a and b, similar to that found in higher plants[81].

Table 1 describes the nutrient composition of an *Ulva lactuca* extract prepared by hot water extraction. The extract exhibits high amounts of nutrients essential for plant growth like potassium, magnesium, phosphorus, calcium and nitrogen. Additionally, the extract also contains high amounts of sodium and chloride, which may be an indication of insufficient removal of salt water from the biomass.

Table 1- Chemical composition of the macroalgae Ulva lactuca *extract*

Adapted from: Gireesh et al.[82]

Chemical parameters ($mg.L^{-1}$)	Macroalgae Extract
Sodium (Na)	185.00
Potassium (K)	113.00
Magnesium (Mg)	108.30
Calcium (Ca)	195.26
Phosporus (P)	51.35
Iron (Fe)	0.37
Chloride (Cl)	415.55
Sulphate (SO_2)	16.84
Silica (SiO_2)	38.12
Cooper (Cu)	0.38
Zinc (Zn)	1.01
Nitrate (NO_3)	19.05

Gupta et al. quantified different plant growth regulators in *Ulva* sp., including gibberellic acid (GA3), indole-3-acetic acid (IAA), abscisic acid (ABA), indole3-butyric acid (IBA), salicylic acid and kinetin riboside (KR). The auxins, IAA and IBA which act as growth regulators were the most prevalent, along with ABA, a stress response regulator. GA3, salicylic acid, and KR were detected in trace amounts [83].

The polysaccharide content in *Ulva* sp. can reach 65%[84]. Ulvans are the most bioactive polysaccharides in this genus, they consist of rhamnose 3-sulfate, xylose, xylose 2-sulfate, glucuronic acid, and iduronic acid units linked by β-(1→4) and α-(1→4) linkage[85]. Ulvan structure is shown in Figure 5.

Ulvan's efficacy as a biotic stress tolerance elicitor is linked to its ability to induce gene expression within the jasmonic acid pathway[86]. Additionally, ulvan increases resistance to fungal pathogens in plants by priming chitin- and chitosan-elicited oxidative burst[87]. Interestingly, oxidative burst and jasmonic acid accumulation are responses to the production of reactive oxygen species[88].

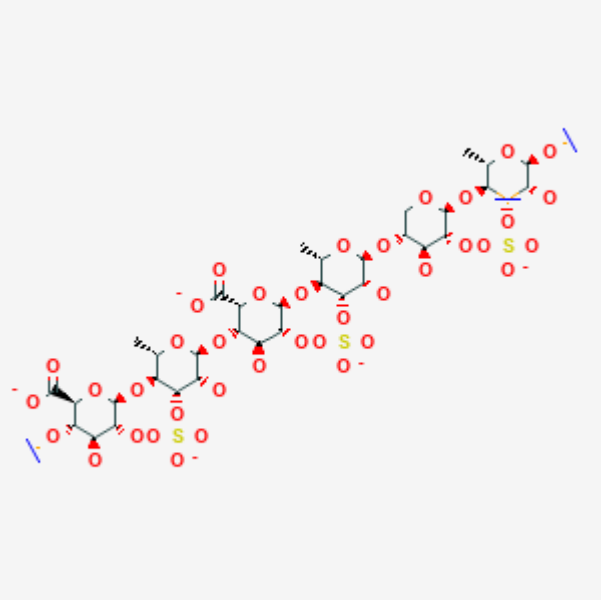

Figure 5- Polysaccharide Ulvan structure

Powdery mildew is the most common disease in *Avena sativa*[89]. The increased resistance against fungal pathogens provided by ulvan in wheat and barley[87] is a good indication that it might have similar effects in oats as all these species belong to the same subfamily, Pooideae. Additionally, an *Ulva armoricana* extract was proven effective against powdery mildew infections in common bean, grapevine and cucumber[90].

Chondrus crispus is an economically important macroalgae from the phylum Rhodophyta. This red macroalgae is widely used as food due to its high content in protein, polyunsaturated fatty acids, minerals, and vitamins[81]. Additionally, *Chondrus crispus* has numerous phytonutrients which give it therapeutic capabilities[91].

The primary economic purpose of *Chondrus crispus* is the extraction of carrageenans. Carrageenans are high molecular weight, sulphated, polysaccharides composed of repeating galactose units and 3,6-anhydrogalactose, linked by alternating α-1,3 and β-1,4 glycosidic bonds[92]. There are six types of carrageenan with slightly different properties which differ from each other in sulphation degree and in the positions of the sulphate group[93]. These polysaccharides find extensive utility in industrial applications as agents for thickening, gelling, suspending, stabilizing, and texturing[94]. Figure 6 illustrates the structure of 3

different carrageenans, κ-carrageenan (20% sulfate content), ι-carrageenan (33% sulfate content), and λ-carrageenan (41% sulfate content)[93].

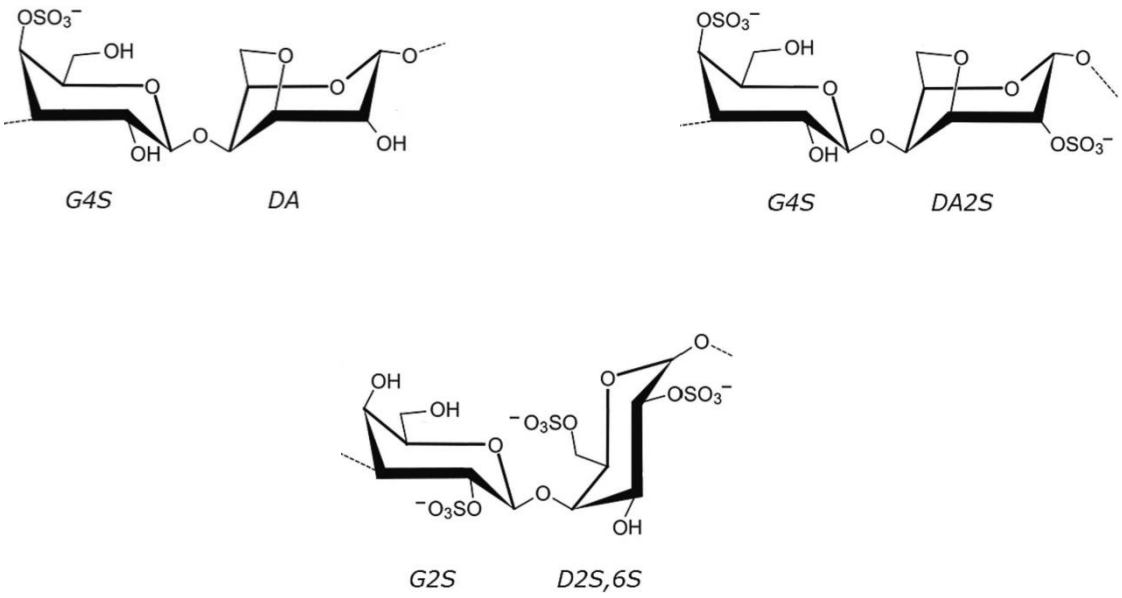

Figure 6- Chemical structure of kappa-carrageenan (left), iota-carrageenan(right) and lambda-carrageenan (bottom)

Carrageenans from red macroalgae elicit growth and resistance to biotic and abiotic stress in multiple plant models[93] through different mechanisms.

In some red macroalgae, including *Chondrus crispus*, the types of carrageenans found vary within different life stages[95]. Remarkably, certain types of carrageenans have been found to act as elicitors of defense responses against *Trichoplusia ni*, a common pest in *Brassica oleracea*, by enhancing the production of glucosinolates[96]. *Chondrus crispus* carrageenans applied in *Brassica napus L.* promote seedling growth and increase germination percentage[95].

In contrast to *Ulva* sp. extracts, complete *Chondrus crispus* extracts haven't captured as much interest as biostimulants, possibly because of the relatively slower growth and lower biomass availability when compared to the most used macroalgae in agriculture. Nevertheless, *Chondrus crispus* bioactive compounds have been shown to enhance oxidative stress tolerance in other organisms[97].

1.5. Motivation and Aim

Given the numerous challenges facing the agricultural market, including fragile supply chains, climate impact, and decreased productivity, macroalgae emerge as potential solutions. This study seeks to explore the biochemical characteristics of two macroalgae water extracts (*Ulva* sp. and *Chondrus crispus*) and assess their effectiveness in addressing these challenges. The objectives of this study include characterizing the extracts to better understand their mechanisms and conducting germination and growth assays in strategically important crops, cabbage and oat, evaluating potential benefits and drawbacks. The results will allow to provide valuable insights for further research in this field.

2. Experimental Section

2.1. Aqueous extraction

Numerous extraction techniques are employed in the production of macroalgae extracts, and each method has distinct consequences for the composition and bioactivity of the resulting extract. Heat, pressure, microwaves, solvents, as well as acids and bases are all utilized in these processes[58,98]. Water-based extractions avoid the use of harmful chemicals and allow for direct use after simple filtration. They are also quite efficient at extracting polysaccharides and phenolic compounds, with mineral extraction increasing as the temperature rises[48,99,100].

2.1.1. Preparation of macroalgal biomass for extraction

The macroalgae *Ulva* sp. and *Chondrus crispus*, were collected in Buarcos Beach, Figueira da Foz in March of 2020 and frozen at -20 ºC until used. The frozen biomass was placed in separate trays and washed 3 times with tap water to remove sand, detritus, and fragments of other macroalgae. Subsequently, the trays were replaced, and the biomass washed twice with distilled water to remove minerals exogenous to the biomass. The trays were then placed in a forced air oven at 50 ºC, until constant weight. The drying process took approximately 48 h.

After drying, the algal material was ground using a coffee grinder and stored in air-tight containers until be used for the extraction.

2.1.2. Water extract preparation

For *Ulva* sp. extract, an Erlenmeyer with 500 ml of distilled water was heated to 90 ºC in a heating plate. Then, 25 g of ground *Ulva* sp. biomass was added and the mixture was kept at 90 ºC for 1 h under magnetic agitation.

For the *Chondrus crispus* extract, 1500 mL of distilled water were used for 25g of ground *Chondrus crispus* biomass. The mixture was kept at 90 ºC for 1 h under magnetic agitation. The extraction apparatus can be seen in Figure 7.

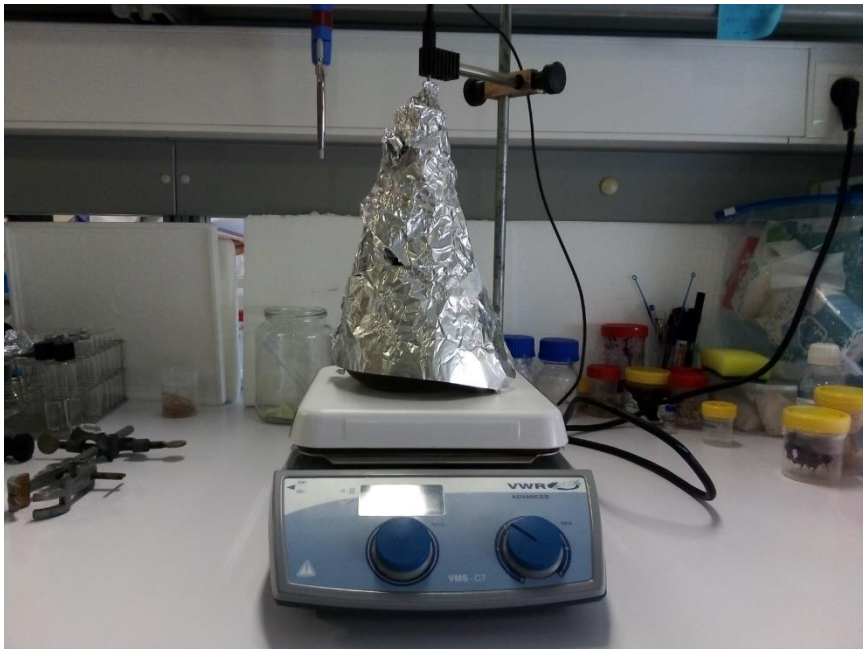

Figure 7- Macroalgae water extraction apparatus

The variation in water content during extraction is attributed to the higher gelation properties of *Chondrus crispus* polysaccharides. When using concentrations higher than the one employed, the suspension becomes excessively viscous, losing its homogeneity.

After the extraction, the solutions were cooled down to ambient temperatures and centrifuged at 14000 rpm to separate the residue from the extracts. Samples of residue and extract were collected and freeze-dried for subsequent biochemical characterization and extraction yield calculation.

The extracts were diluted to a concentration of 1 mg/mL. Subsequently, their conductivity (portable conductivity meter: ProfiLine Cond 3310 WTW, Oberbayern, Germany), pH (pH meter: 3310 Jenway, Staffordshire, UK), and viscosity (ROTAVISC lo-vi, IKA, Germany) (Figure 8) were measured before being stored at a temperature of -20ºC. The *Ulva* sp. extract obtained was called UE and the *Chondrus crispus* extract obtained was called CE.

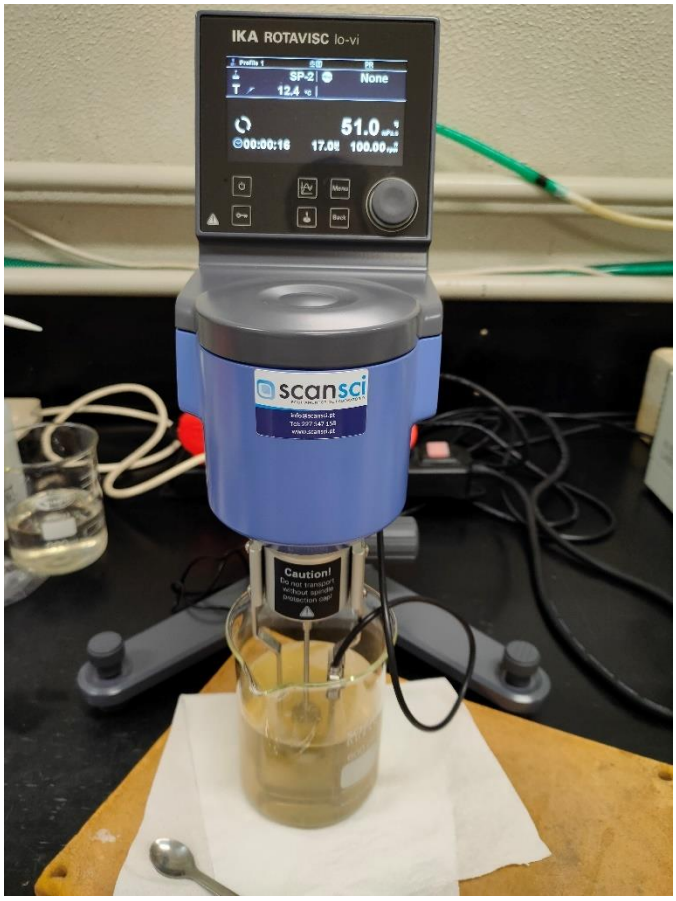

Figure 8- Chondrus crispus extract viscosity measurement.

2.2. Extract Biochemical Analysis

For the biochemical analysis of the extracts, the freeze-dried samples were used in the different procedures.

2.2.1. FTIR-ATR analysis

The Fourier-Transform Infrared Spectroscopy - Attenuated Total Reflection (FTIR-ATR) analysis is a rapid method for the characterization of polysaccharides and other compounds. The analysis was based on the protocol described by Pereira et al. (2013). [103]

For FTIR-ATR analysis, the freeze-dried samples from the macroalgae extracts were subjected to direct analysis (spectrometer: ALPHA II Compact FT-IR Spectrometer, Bruker, Germany) without any further preparation. All spectra obtained are the average of two independent measurements from 400 to 4000 cm^{-1} with 24 scans, each at a resolution of 4 cm^{-1}.

2.2.2. Elemental Analysis

The elemental analysis of the extracts was conducted by the elemental analysis service at the Department of Chemistry of University of Aveiro.

Sample Size was approximately 1 mg for each replicate, two replicates of each analysis were made.

The equipment used was a Truspec 630-200-200, Combustion Furnace Temperature was 1075 °C, and Afterburner Temperature was 850 °C.

The detection methods were: Infrared Absorption for carbon, hydrogen, sulfur, and thermal conductivity for nitrogen.

2.2.3. Mineral Analysis

In an external laboratory, mineral concentrations of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), copper (Cu), manganese (Mn), iron (Fe), and zinc (Zn) were determined using inductively coupled plasma optical emission spectrometry (ICP-OES) on a Horiba JobinYvon Activa M spectrometer. The method had a detection limit of 10µg/L with an experimental range of error of 5%.

Prior to analysis, 200 mg of solid samples were digested using 1 mL HNO_3, 2 mL H_2O_2, and microwave heating at 180 °C.

2.2.4. Neutral sugar analysis

In *Ulva* sp., neutral sugar analysis followed a procedure adapted from Coimbra et al. (1994) [98].

Two milligrams of each sample, macroalgae biomass, water extract, and residue, were accurately weighed and placed in individual culture tubes. Subsequently, 200 µL of a 72% H_2SO_4 solution was added to each tube. The resulting mixture was incubated at room temperature for 3h, with intermittent agitation.

Following the incubation period, 2.2 mL of H_2O was added, resulting in a final H_2SO_4 concentration of 1M. The hydrolysis reaction was carried out at 100°C using a heating block for 1 h. Once completed, the tubes were cooled in an ice bath. Subsequently, 0.5 mL of the hydrolysate was transferred to a sample holder for posterior uronic acid analysis. The hydrolysis reaction continued for another 1 h and 30 min.

For the analysis of monosaccharides, 200 µL of the internal standard (2-deoxyglucose 1 mg/mL) was added, and the acid neutralized using 200 µL of NH_3, then, the monosaccharides were reduced using 100 µL of a 15% $NaBH_4$ solution in a 3 M NH_3 environment at a temperature of 30 ºC for 1 h. The excess of BH_4^- was eliminated using 100 µL of glacial acetic acid, and 300 µL were transferred to sovirel tubes.

Afterward, the samples were acetylated with 450 µL of 1-methylimidazole and 3 mL of acetic anhydride following incubation at 30 ºC for 30 min.

In *Chondrus crispus*, the reductive hydrolysis method proposed by Stevenson et al. (1991) [99] was used. This method is indicated for the analysis of sulphated galactans from red algae.

One milligram of each sample, macroalgae biomass, water extract, and residue, was accurately weighed and transferred into speedvac tubes. Subsequently, each sample was dissolved in 200 µL of 3 M TFA (Trifluoroacetic acid), and 50 µL of MMB (Borane 4-methylmorpholine) was added. The tubes were then subjected to a 5 min incubation at 80°C.

After cooling to room temperature, an additional 50 µL of MMB at 80 mg/mL was added. The tubes were incubated at 120 °C for 1 h. Following this, the tubes were cooled, 100 µL of MMB and 100 µL of the internal standard (2-deoxyglucose 1 mg/mL) were added. The solvents were evaporated under reduced pressure. The residue was dissolved in 100 µL of glacial acetic acid. Afterward, the samples were acetylated with 450 µL of MMB and 3 mL of acetic anhydride following incubation at 30 ºC for 30 min.

Alditol acetate derivatives obtained from both methods were separated using dichloromethane in the following manner, in an ice bath, 3 mL of distilled water were added to break down excess acetic anhydride, and 2.5 mL of dichloromethane were introduced to extract alditol acetates. After agitation, the samples were centrifuged, and the aqueous phase was removed. This process was repeated twice. The organic phase was washed by adding 3 mL of distilled water, followed by agitation, centrifugation, and complete removal of the aqueous phase. This step was also repeated twice. Dichloromethane was evaporated under reduced pressure. 1mL of anhydrous acetone was added, and the evaporation process was repeated to ensure dryness.

The samples were analyzed using a Perkin Elmer – Clarus 400 chromatography system equipped with a flame ionization detector (GC-FID). The GC column utilized was a DB-225 with dimensions of 30 meters in length, 0.25 mm inner diameter, and a 0.15 µm film thickness. The temperature profile involved an initial heating step to 200 °C, followed by a linear temperature increase of 40 °C per minute until reaching a temperature of 220 °C, which was maintained for 7 min. Then, 20 ºC per minute until reaching 230 ºC which was maintained for 1 min. Monosaccharides were identified by comparing their retention times with standards. Sugar quantification was conducted by comparing the chromatographic peaks of sugars with those obtained for the internal standard, 2-deoxyglucose (Merck KGaA, Darmstadt, Germany).

2.2.5. Uronic Acids Analysis

Uronic acids quantification followed a procedure adapted from Coimbra et al. (1997) and Selvendran et al. (1979) [98,100]. Samples were first subjected to hydrolysis as described in section 2.2.1, Galacturonic acid (GalA) standards were prepared in the 0-100 µg/mL concentration range with the purpose of establishing a calibration curve.

For each standard/sample, three test tubes were prepared, including one blank and two replicates, each containing 0.1 mL of either GalA standards or samples.

The tubes were placed in an ice bath, and then 1 mL of 200 mM boric acid in concentrated sulfuric acid was added to each tube. Then, the tubes were subjected to a 100 °C bath for 10min, afterward, they were cooled in an ice bath.

In two of the three tubes containing standards and samples, 100 µL of MFF (m-phenylphenol 0.15% w/v in 0.5% w/v NaOH) were added in low light. The remaining tube without MFF served as the blank for analysis. The tubes were manually agitated, and the absorbance was measured at 520 nm using a microplate spectrophotometer (Biotek EUN).

2.2.6. Total phenolic compounds: Folin-Ciocalteu method

The concentration of total phenolic compounds (TPC) in the macroalgae extracts was estimated using the Folin-Ciocalteu method. According to the procedure described by Touati et al. (2015) [105].

A solution of Sodium Carbonate (Na_2CO_3) 0.07 g/mL was prepared, and gallic acid standards in the 0.0250-0.00125 mg/mL concentration range were prepared with the purpose of establishing a calibration curve.

Three replicates were done for each sample or standard, in each well, 30 µL was added, along with 150 µL of diluted Folin's reagent. Then, the microplate was incubated for 5 min.

Next, 150 µL of Na_2CO_3 solution was added and the microplate was incubated inside the microplate reader for 60 min at 30 ºC, then, the absorbance at 760 nm was read.

The results were expressed in grams of gallic acid equivalents (g GAe /g sample).

2.2.7. Antioxidant activity: ABTS

The antioxidant activity was evaluated with the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical discoloration method. The procedure was adapted from Re et al. (1999) [101].

An ABTS 7 mM in potassium persulfate 2.45 mM solution was prepared. The ABTS solution concentration was adjusted to an absorbance between 0.750 and 0.800 at 730 nm using a microplate spectrophotometer (Biotek EUN). Ascorbic acid standards in the 0.0250-0.00125 mg/mL concentration range were prepared with the purpose of establishing a calibration curve. The samples were diluted to fit in the 0.200-0.800 absorbance range.

For each standard/sample, four wells were prepared, including one blank and three replicates, each containing 50 µL of either Ascorbic acid standards or samples. In standard/sample wells, 250 µL of ABTS solution was added, in blank wells, 250 µL of solvent was added. The samples were incubated in the dark for 20 min at room temperature before measuring the absorbance at 730 nm.

The results were expressed in grams of ascorbic acid equivalents (g AAe /g sample).

2.3. Seed Germination assay

For the seed germination assay, a procedure described by Pacheco et al. (2021) [107] was followed, cabbage and oat seeds from commercial suppliers, Semillas Batlle S.A. and Agrovete, Lda, respectively, were selected for uniformity, after selection the seeds underwent a sterilization process. Initially, they were immersed for one minute in a 2% sodium hypochlorite solution and then for three minutes in distilled water. Sterilized Petri

dishes (15cm × 15cm) were then prepared with cotton and filter paper, into which 70 mL of each macroalgae extract was added. A control group was established with the addition of an equal volume of distilled water.

Next, 25 disinfected seeds of cabbage were sown on the filter paper within each Petri dish (Figure 9). These dishes were then incubated (Heraeus B5090E Incubator, Thermo Scientific, Osterode, Germany), at a constant temperature of 22 ± 1°C and kept in darkness for 21 days in cabbage seeds case and 7 days in oat seeds case (Figure 10). Each of these procedures was conducted in triplicate to ensure the reliability of the results.

Following the germination period, the number of germinated seeds was counted, and the germination percentage (GP) was calculated using the formula:

$$\mathrm{GP} = \frac{\text{number of germinated seeds}}{\textit{total number of seeds}} \times 100$$

Furthermore, plant growth parameters were assessed for all seedlings. These parameters included the measurement of the aerial part (from the base of the hypocotyl to the apical bud) and radicular length of the longest root using a ruler (Shatterless 75 S.50, Molin, Portugal). The fresh weight of the aerial and radicular parts was determined using an analytical scale (PC2000 Mettler-Toledo, Zurich, Switzerland).

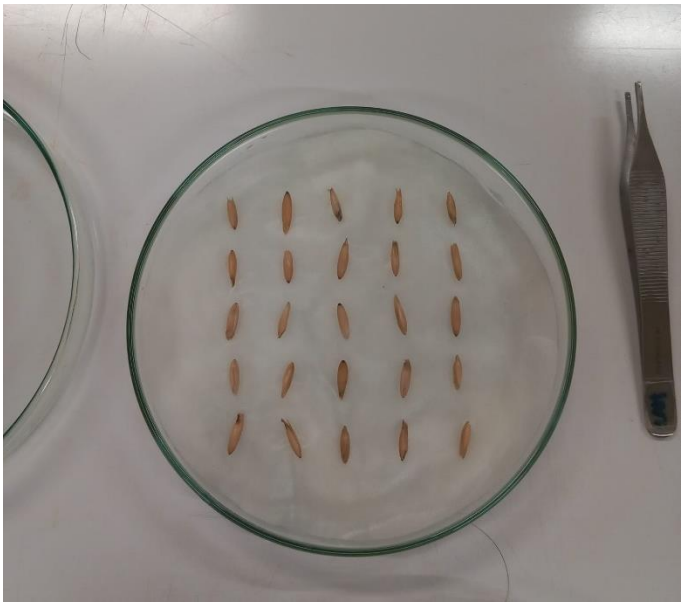

Figure 9- Oat seed disposition in Petri dish

Figure 10- Germination assay Petri dishes in the incubator

2.4. Juvenile Growth assay

For the juvenile growth assay, cabbage seeds and oat seeds from commercial suppliers, Semillas Batlle S.A. and Agrovete, Lda, respectively, were selected for uniformity. Two seeds were placed in each slot of a germination tray filled with germination substrate from Semillas Batlle S.A. In total, 39 slots (78 seeds) were used for each treatment. A control group was established with an equal number of seeds.

The germination tray was positioned inside a seed starter kit, which was filled with tap water until the germination tray floated, and subsequently closed (Figure 11).

Throughout the first 7 days, a constant temperature of 22 ± 1ºC was maintained, with humidity levels at 83 ± 3%. The photoperiod was set at 16 hours of light and 8 hours of darkness, with an illumination intensity of 2000 lux.

After 7 days, the seed starter kit was opened. Each group of juvenile plants was isolated from the others using plastic sheets. Then, each group was subdivided into three equal portions, and each one-third segment was subjected to 1 round of pulverization with the specified

treatment (Figure 12). The control group was pulverized using distilled water, each pulverization round amounted to approximately 4mL.

After another 7-day period under the same conditions, plant growth parameters were assessed for all plants. These parameters included the measurement of the aerial part (from the base of the hypocotyl to the apical bud) and radicular length using a ruler (Shatterless 75 S.50, Molin, Portugal). The fresh weight of the aerial and radicular parts was determined using an analytical scale (PC2000 Mettler-Toledo, Zurich, Switzerland).

Figure 11- Seed starter kit for the juvenile growth assay

Figure 12- Macroalgae extract foliar application.

2.5. Plant Biomass Biochemical Analysis

The aerial and radicular sections of each treatment (*Ulva* sp. extract, *Chondrus crispus* extract, and control) were collected in separate petri dishes, and then, the biomass was dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) during 48 h at 60 °C.

The dry weight of the biomass was measured using an analytical scale (Kern, Germany).

The aerial biomass was then ground using a Mortar and pestle (Figure 13), in the cases where cellulose fiber difficulted the process, the fibers were first frozen with liquid nitrogen. The ground biomass was collected and stored in air-tight sample holders for posterior biochemical analysis.

This procedure was done for seed germination and juvenile growth assays.

Figure 13- Biomass griding process

2.5.1. Extraction procedure

The dried biomass collected after the germination assay was not enough to preform individually all the biochemical analysis proposed, to address this limitation, a cascade biorefinery approach was followed in which different fractions were collected from the same initial biomass.

The procedure was adapted from Pardilhó et al. (2023) [108], in the original procedure, 1 g of algal biomass is used. In the present work was experimented with a sample 100 times smaller and a different type of biomass, plant biomass instead of algal biomass.

The initial step involved extracting non-polar compounds, primarily lipids. In this process, 10 mg of powdered sample was submerged in 400 µL of n-hexane (at a ratio of 1:20 weight-to-volume) and subjected to ultrasound treatment for 20 min at room temperature. The n-hexane was then collected. This procedure was repeated until a colorless extract was achieved. The obtained extracts were combined, and the n-hexane was removed under reduced pressure leaving only the oil. This fraction was used for lipids analysis.

The remaining solid portion underwent extraction of polar compounds, following the same protocol, but with methanol as the solvent. The obtained extracts were combined, this fraction was used for pigment quantification and antioxidant activity analyses.

In the final stage, the solid residue resulting from the previous extractions was immersed in 2 mL of distilled water and heated to 100 °C for 2 h. This final extract was retrieved through centrifugation, and the present polysaccharides were precipitated by adding ethanol in a 1:3 volume ratio and stored in a cold environment. After 24 h, the solution was centrifuged for 10 min at 4000 rpm and the solvents were removed under reduced pressure. The solid polysaccharides were collected for polysaccharide analysis.

2.5.1.1. Lipids characterization

An internal standard (methyl heptadecanoate, C:17 0.3 mg/mL) was prepared in cyclohexane, as well as a solution of KOH 2 M in methanol and a saturated solution of NaCl.

Two hundred micrograms of internal standard were added to the samples. The Fatty Acid Methyl Esters (FAMEs) were obtained by transesterification: Twenty microliters (20 µL) of KOH solution was added to each sample. The vial was then sealed and vigorously shaken for 30 s on a vortex shaker. Then, 0.2 mL of the saturated NaCl solution was added. The solution was subsequently centrifuged at 2000 rpm for 5 min. Following centrifugation, the organic phase was transferred to another vial and an aliquot was utilized for GC analysis.

The FAMEs in each plant biomass sample were separated and quantified through gas chromatography coupled with mass spectrometry (GC-MS), using an Agilent Technologies 6890 N Network (Santa Clara, CA). This equipment has a 0.25 mm internal diameter, 0.1 µm film thickness and 30 m long DB-FFAP column. The temperature profile involved an initial heating step to 75 °C, following a linear temperature increase of 15 °C min^{-1} to 155 °C, followed by another temperature ramp of 3 °C min^{-1} to 180 °C and finally an increase of 40 °C min^{-1} until a final temperature of 220 °C which was maintained for 3 min.

FAMEs were identified by comparing their retention times with standards. FAME quantification was conducted by comparing the chromatographic peaks of FAMEs with those obtained for the internal standard, methyl heptadecanoate, C:17.

2.5.1.2. Antioxidant activity: ABTS

The antioxidant activity of the methanolic fraction of the extraction procedure was determined following the same procedure as in section 2.2.3.

2.5.1.3. Pigments Qualification and Quantification

The methodology for pigment detection relied on the approach outlined by Cotas et al. [109]. This technique involves employing thin-layer chromatography (TLC) to segregate and ascertain the pigment composition within methanolic extracts. Spectrophotometry is then utilized for both quantitative and qualitative assessment of these pigments.

Pigments were identified using a silica gel TLC plate (ALUGRAM Xtra SIL G UV254, supplied by Macherey-Nagel in Germany) which was activated by heating at 120 ºC for 5 min using an air-forced oven (Raypa DAF-135, Spain). Subsequently, 20 µL of each extract (methanol:acetone 1:1) was applied. The plate was developed in a chromatography chamber using an eluent comprised of petroleum ether and acetone in a 7:3 v/v ratio until the front reached a height of 10 cm (Figure 14). Following this, the plate was removed, and the solvent was allowed to evaporate at room temperature.

To identify the pigments, the retention factor (Rf), which involves determining the compound's migration distance and comparing it to the distance travelled by the eluent, was calculated and compared to the existing literature.

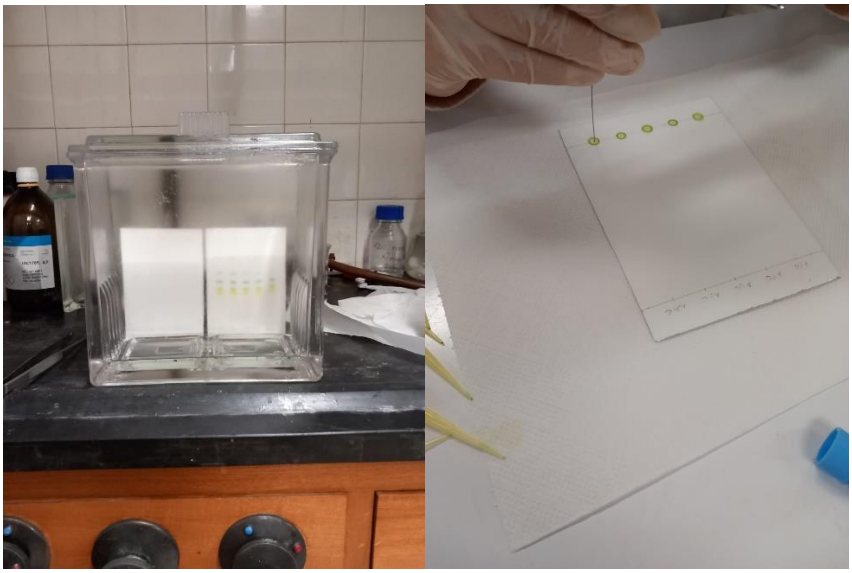

Figure 14- Thin layer chromatography (TLC) methodology

Pigment quantification was carried out using UV-Vis spectroscopy. For each methanolic sample, absorbance measurements were taken at four specific wavelengths: 470 nm, 535 nm, 652 nm, and 665 nm. It's important to note that each measurement was performed individually. This was necessary because methanol has a low boiling point, and if too much time had elapsed between measurements, the results could be affected due to variations in pigment concentration.

These measurements were used to quantify Chlorophyll a and b, carotenoids, and anthocyanins.

Chlorophyll a and b, carotenoids quantification was done with the formulas provided by Toscano et al.[110]:

Chl a = 16.75A665 − 9.16A652
Chl b = 34.09A652 − 15.28A665
Carotenoids = (1000A470 − 1.63Chl a − 104.96Chl b)/221

Total Anthocyanins was calculated by the formula by Lao et al. [111]:

$$\text{Total anthocyanins } \left(\frac{\text{mg}}{100}\text{g}\right) = \frac{100 \times \text{A} \times \text{DF} \times \text{V}}{98.2 \times x}$$

Where,

100/ is a constant that takes the extinction coefficient and unit conversions into consideration

98.2 unit conversations into consideration

A absorbance of sample at 535 nm

DF is the dilution factor

V is the known volume pigment extract was made up to after extraction (ml)

x is the weight of biomass sample (g)

2.5.1.4. Neutral sugar analysis

For plant biomass sugar analysis, a procedure adapted to small samples was followed, 1 mL of 2 M TFA solution was added to each sample and hydrolysis was carried out in a heating block at 120 °C for 1 h. The acid was evaporated to dryness under reduced pressure, and 20 µL of internal standard (2-deoxyglucose) was subsequently added.

Then, the monosaccharides were reduced using a 15% $NaBH_4$ solution in a 3 M NH_3 environment at a temperature of 30 ºC for 1 h. Following reduction, glacial acetic acid was added to neutralize excess BH^-_4. Subsequently, the samples were acetylated with acetic anhydride and the alditol acetate derivatives were separated using dichloromethane and analyzed by GC-FID in the same conditions as stated in section 2.2.1.

2.5.2. Uronic acids analysis

Plant biomass uronic acids quantification followed the procedure as described in section 2.2.2.

2.5.3. Elemental Analysis

Elemental analysis was determined with ground plant biomass (Cabbage and Oat aerial sections) following the same procedure as described in section 2.2.5.

2.5.4. FTIR-ATR analysis

The dried plant biomass samples were analyzed by FTIR-ATR following the same procedure as described in section 2.2.7.

2.6. Statistical analysis

Statistical evaluation of the growth parameters was carried out using the software Sigma Plot v.14. To ensure the validity of the data, normality was assessed using the Shapiro–Wilk test, and homogeneity with the Brown-Forsythe equal variance test. In cases where the normality test was rejected, the Holm-Sidak method was applied for subsequent analysis. One-way analysis of variance (ANOVA) was then executed to ascertain the presence of statistically significant disparities among the growth parameters of the different treatments. Comparisons were made between different treatments, and statistical significance was determined at a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Extracts characterization

Following *Ulva* sp. and *Chondrus crispus* aqueous extraction, the extracts physical characteristics were measured, and multiple biochemical analysis were conducted in order to characterize the samples.

3.1.1. Extraction yield

Ulva sp. extraction yield, 27.95% (w/w), was higher than *Chondrus crispus* extraction yield, 16.75% (w/w). The difference is most likely caused by *Chondrus crispus* high carrageenan content, this polysaccharide has high gelification properties, there was noticeable difference between the extract residue of each macroalgae. *Chondrus crispus* extraction residue was a gel which may have trapped *Chondrus crispus* components in the residue. To address this issue the extract would need to be filtrated at high temperatures to avoid gelification, approximately 90ºC, with specialized equipment to address safety considerations.

3.1.2. Extracts Physical Properties: conductivity, pH and viscosity

The physical properties of the macroalgae extracts are listed in Table 2.

Table 2- Physical properties of the extracts of macroalgae Ulva sp. *and Chondrus crispus extracts.*

	Ulva sp. extract	*Chondrus crispus* extract	Distilled Water
pH	6.28	6.27	5.56
Viscosity (mPa.s)	7.5	51	-
Conductivity ($\mu S.cm^{-1}$)	95	428	6

Electrical conductivity (EC) is an indirect measure of salinity. High EC can adversely affect seed germination by decreasing water absorbency and disrupting metabolic pathways, most

crops require EC below 400 μScm^{-1} for ideal germination, at higher levels yields can be reduced[112]. At 1 mg/mL concentration, *Ulva* sp. extract (*UE)* is comfortably below the 400 μScm^{-1} threshold, in contrast, the *Chondrus crispus* extract (*CE)* is slightly above the threshold, although at levels where normal germination is still expected.

The pH of *Ulva* sp. and *Chondrus crispus* extracts were 6.28 and 6.27 respectively, both likely influenced by the pH of the distilled water used in the extractions and subsequent dilution.

Neutral pH values are generally preferred for seed germination, significant variation from neutral pH can affect mineral absorption by seedlings[107]. However, in some cases, slightly acidic media can be preferred. One study pointed the 6.2-6.4 pH interval is preferred for B*rassica oleracea* microspore embryogenesis[113].

Accessing the viscosity of the extracts applied is relevant for the industrial application prospects, in industrial-scale agriculture, liquid fertilizers and pesticides are applied by machines called sprayers. These machines use nozzles to spray the liquid being used, if the viscosity is too high the spray area is diminished, and homogenous application is not assured[114,115].

Lechler, one of the leading nozzle suppliers recommends a maximum viscosity of 100 mPas in for low-pressure applications [115,116]. Water viscosity is approximately 1 mPas. However, at such low viscosities, the issue of pesticide drift, the movement of pesticides to nontarget areas, becomes quite prevalent. To address this problem, drift retardants are employed to augment the viscosity of the spray solution. This, in turn, reduces solution loss and helps in mitigating environmental pollution in the surrounding area[114].

Ulva sp. and *Chondrus crispus* extracts viscosity were measured at 7.5 mPas and 51.0 mPas, respectively. Both can be used in agricultural systems although parameters like spray pressure, spray height, or nozzle type may need to be adapted to each extract.

3.1.3. FTIR-ATR extract analysis

FTIR-ATR spectra of *Ulva* sp. and *Chondrus crispus* extracts exhibit several similarities (Figures 15 and 16). The bands at 3500–3200 cm^{-1} correspond to the O-H stretching of hydroxy groups, while the bands at 3000–2800 cm^{-1} correspond to C–H stretching, characteristic of polysaccharides.

Both spectra indicate the presence of proteins as these structures induce various signals between 1550 cm^{-1} and 1650 cm^{-1}. Uronic acid carboxyl groups also vibrate in this range, as well as around 1420 cm^{-1}[117].

FTIR-ATR Spectra – *Ulva* sp. Extract

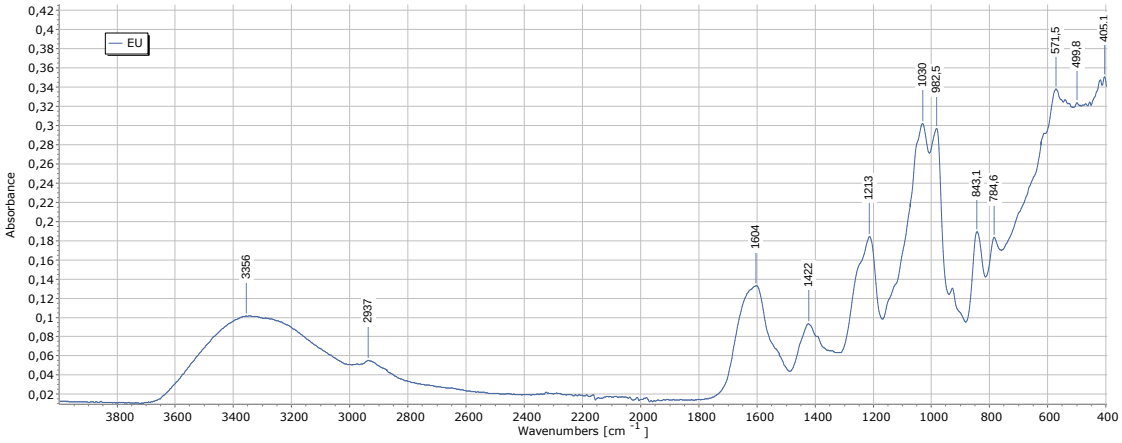

Figure 15- FTIR-ATR Spectra of Ulva sp. *extract*

FTIR-ATR spectra of *Ulva* sp. extract (Figure 15) shows several similarities to the Ulvan spectra documented in the literature. The peak at 1030 cm^{-1} corresponds to symmetric stretching of C-O-C linkages. The peak at 1213 cm^{-1} corresponds to S=O stretching of sulfate groups and the peak at 843.1 cm^{-1} is characteristic of C-O-S stretching, suggesting the presence of ulvan sulphate groups[117].

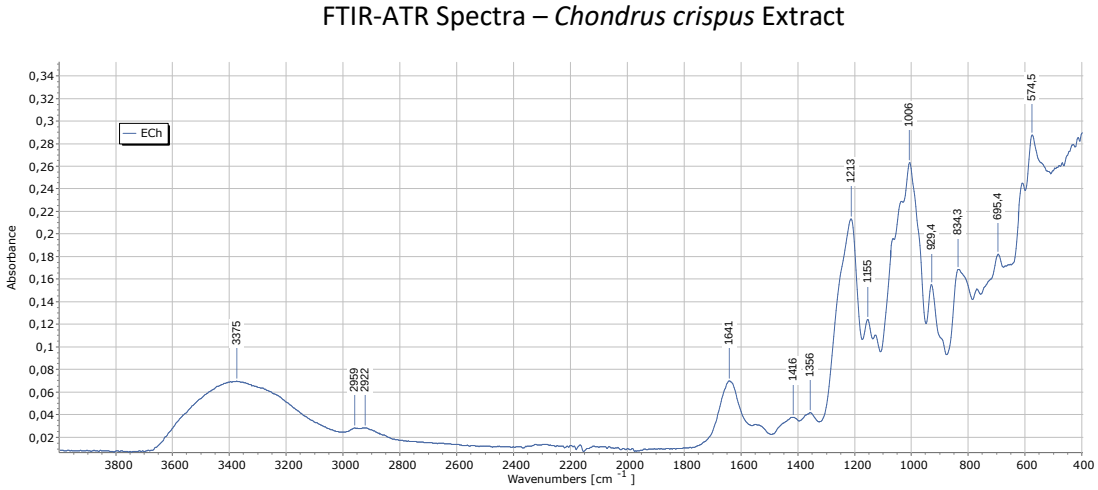

Figure 16- FTIR-ATR Spectra of Chondrus crispus *extract*

FTIR-ATR spectra of *Chondrus crispus* extract (Figure 16) exhibits several similarities with carrageenan spectra found in the literature[95]. Specifically, peaks were observed at 1213 and 1155 cm^{-1}, which can be attributed to sulphated esters, while the peak at 929.4 cm^{-1} corresponds to the C-O bond in 3,6-anhydrogalactose. Additionally, the peak at 1006 cm^{-1} can be attributed to the C-O and C-C stretching vibrations of the pyranose ring, and the peak at 929.4 cm^{-1} is associated with the C–O bond of 3,6-anhydrogalactose. Furthermore, the 834.3 cm^{-1} peak can be linked to the C–O–SO_3 bond on C2 of galactose, further confirming the presence of sulphated polysaccharides. Lastly, the peak at 695.4 cm^{-1} corresponds also to 3,6-anhydro-L-galactose.

It is worth noting that some variability in *Chondrus crispus* spectra is expected, as the chemical composition of algae and, consequently these spectra, can be influenced by the life cycle stage of the biomass[95].

3.1.4. Elemental and mineral composition

The results of the elemental analysis are shown in Table 3. The results show high carbon and hydrogen percentages, indicating a prevalence of organic compounds, such as carbohydrates and proteins in the macroalgae extracts.

Table 3- Elemental composition of Ulva sp. *and* Chondrus crispus *extracts*

	Elemental Composition (%)			
Extract	C	H	N	S
Ulva sp.	28.671	4.906	2.068	3.9785
Chondrus crispus	23.536	3.741	1.2695	6.3955

As a fertilizer, nitrogen plays a crucial role in the synthesis of plant hormones, proteins, and the production of chlorophyll[118]. The extracts nitrogen percentage indicates the presence of not only protein but also chlorophyll, free amino acids and inorganic nitrogen[119].

Using the nitrogen-to-protein conversion factor for macroalgae (5)[119], the protein content was estimate of approximately 10.3% in *UE* and 6.3% in *CE*. Pereira et al. [120] reported that the protein content in *Ulva lactuca* falls within the range of 10-25%, and in *Chondrus crispus*, it ranges from 11-21%. This suggests that the protein extraction was more effective in the *UE* compared to the *CE*, consistent with the differences in total extraction yield we observed.

Both extracts have high content in sulfur. Sulfur plays a crucial role in plant biology as it is a key component of amino acids in plant proteins and is vital for energy production[118]. Sulfur is also related to the amount of sulphate groups present in ulvan and carrageenan. The *CE* contains a higher percentage of sulfur. According to the literature, the bioactivity of macroalgae polysaccharides is positively correlated with the degree of sulfation[43].

The results from the mineral analysis are stated in Table 4. The mineral analysis of *Ulva* sp. and *Chondrus crispus*, both in biomass and extract forms, demonstrates notable differences in their mineral content. Consistent with *Ulva* sp. biomass, its extract is a rich source of magnesium and manganese, crucial in the photosynthetic process[118]. In contrast, *Chondrus crispus* biomass and extract have high content in potassium, sodium, and zinc, involved in enzyme function and growth[118].

Table 4- Mineral composition of Ulva sp. *and Chondrus crispus biomass and respective water extracts*

Samples	Ca (g/kg)	K (g/kg)	Mg (g/kg)	Na (g/kg)	P (g/kg)	Cu (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Zn (mg/kg)
Ulva sp. Biomass	8.4	1.8	18.6	1.2	1.4	3.9	104.9	1261.3	18.8
Chondrus crispus Biomass	5.8	20.2	8.2	26.0	1.7	2.7	25.3	182.6	200.6
Ulva sp. Extract	16.2	1.7	29.7	2.8	0.8	1.2	156.1	17.5	29.3
Chondrus crispus Extract	7.0	23.7	8.8	33.6	1.8	1.8	24.4	91.6	124.8

Copper content, an enzyme constituent[118], is variable, with *Ulva* sp. biomass having a higher copper content (3.9 mg/kg) than *Chondrus crispus* biomass (2.7 mg/kg). However, in extract form, *Chondrus crispus* (1.8 mg/kg) surpasses *Ulva* sp. (1.2 mg/kg) in copper. Phosphorus content remains relatively low and similar between the two biomass types and their extracts. Calcium content is higher in *UE* compared to *CE*.

The overall mineral quantity in *CE* is higher than that of *UE*, partially explaining its extract's higher electrical conductivity. *CE* high sodium content is a potential concern, most plants sodium requirements are low and its presence in high concentration may interfere with the absorption of water and other minerals[121].

In a general manner, the mineral composition of these extracts is complimentary, from a fertilizer perspective; the junction of these two macroalgae provides a more complete mineral profile.

3.1.5. Sugar Composition

The results of the sugar analysis of *Ulva* sp. biomass, water extract, and residue are represented in figure 17. The *Ulva* sp. extraction residue contains a high total percentage of sugars, totaling 71.54%, while the *UE* has 53.09%.

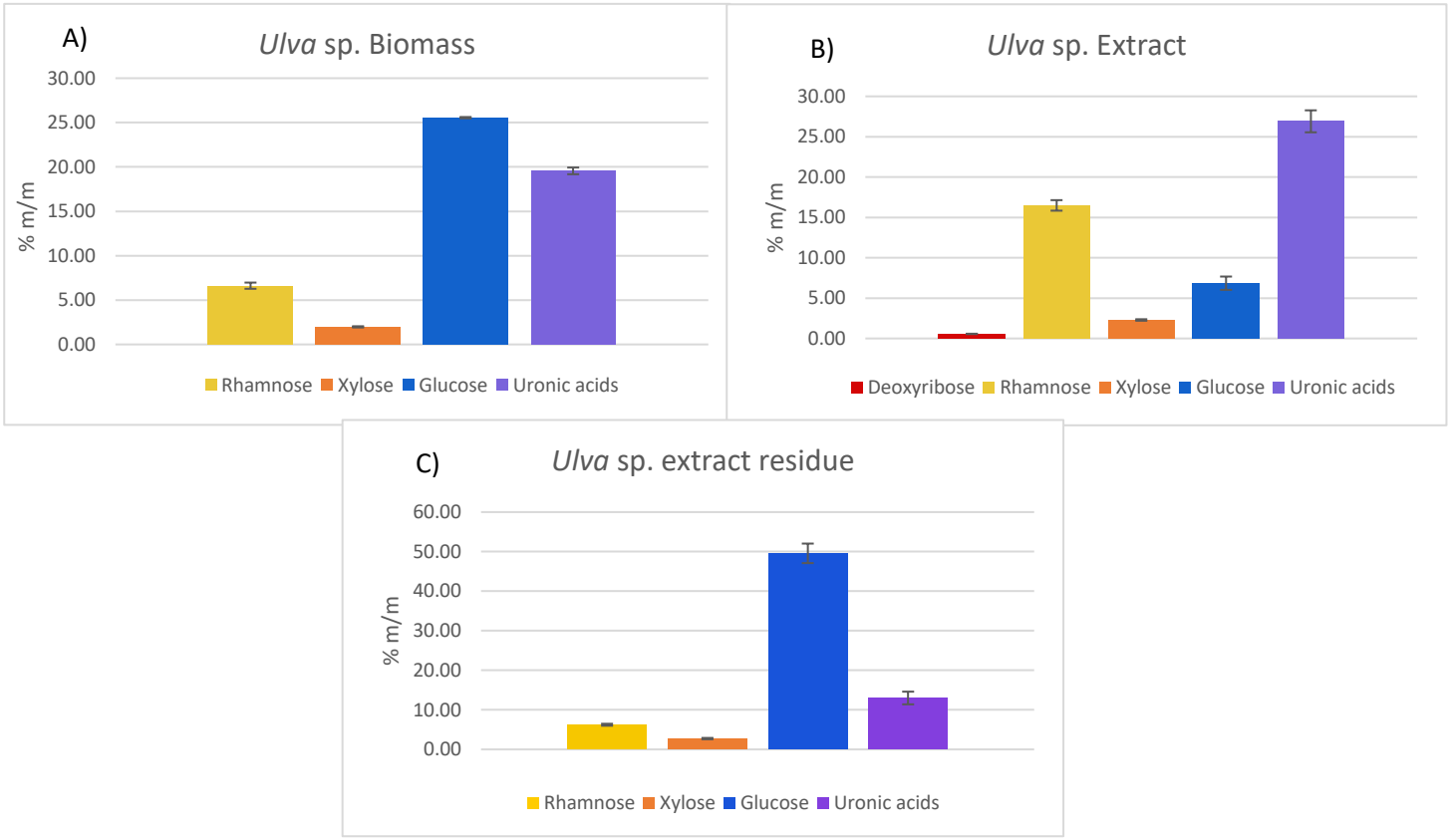

Figure 17- Sugar analysis of Ulva sp.*: Biomass (A), Extract (B) and Extraction residue (C)*

The most abundant sugar in the original biomass is glucose, making up 25.55± 0.07 % (m/m) of the composition. Glucose is the primary component of green algae cell walls in the form of cellulose, an insoluble fiber[81]. As expected, after extraction, the glucose content decreases in the extract (6.84 ± 0.83 % (m/m)) and substantially increases in the residue (49.57 ± 2.47 % (m/m)). This suggests that most of the glucose is in the form of cellulose and is discarded during the aqueous extraction process.

Uronic acids are constituents of various polysaccharides [117], including ulvan. The content of these monosaccharides in the original biomass was 19.56 ± 0.38 % (m/m). In the extract,

the concentration of uronic acids increases to 26.90 ± 1.36 (m/m) %, while the residue still retains a significant amount at 13.00 ± 1.60 % (m/m). In the original biomass, rhamnose, another component of ulvan, comprises about 6.62± 0.35 % (m/m) of the composition (Figure 15A). After extraction, the extract contains a higher concentration of rhamnose (16.49 ± 0.65 % (m/m)) compared to the original biomass (Figure 15B). This increase in uronic acids and rhamnose in the extract indicates that ulvan is soluble and has been extracted to a significant extent. In contrast, xylose is present at a relatively low level in *Ulva* sp. biomass, around 1.98± 0.07 % (m/m), and this remains low both in the extract and residue. It is interesting to note that xylose is also a constituent of ulvan.

The results of the sugar analysis of *Chondrus crispus* biomass, water extract, and residue are represented in figure 18.

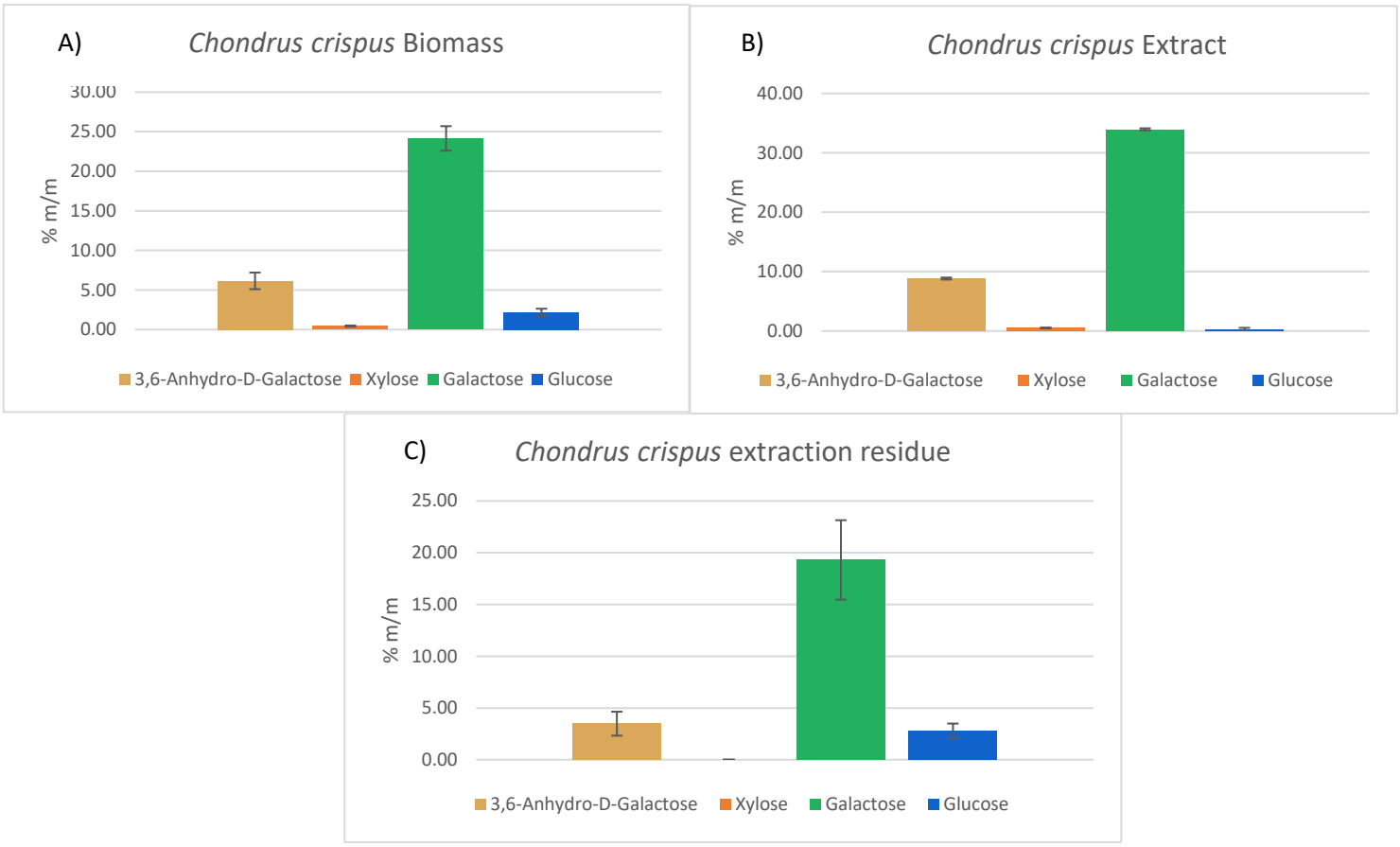

Figure 18- Sugar analysis of Chondrus crispus Biomass (A), Extract (B) and Extraction residue(C)

Chondrus crispus samples were subjected to reductive hydrolysis to permit the quantification of 3,6-anhydro-D-galactose, a component of the carrageenans constituting *Chondrus crispus* cell walls[99]. This sugar is present in the original biomass at 6.16 ± 1.05 % (m/m). In the water extracts, the content of 3,6-anhydro-D-galactose increases in the extract to 8.83 ± 0.17 % (m/m) and decreases in the residue to 3.48 ± 1.16 % (m/m). A similar trend is observed with galactose, the dominant sugar in the original biomass at 24.1 ± 1.55 % (m/m). After extraction, the galactose content in the extract increases to 33.9 ± 0.18 % (m/m) and decreases in the residue to 19.3 ± 3.84 % (m/m). The data indicates efficient carrageenan extraction which explains the extract relatively high viscosity when compared to *UE* as described above in section 3.1.2.

Glucose is present at 2.17 ± 0.47 % (m/m) in the original biomass but only in very low amounts in the extract 0.27± 0.27 % (m/m). The residue retains most of the glucose (2.79 ± 0.70 % (m/m)) present in the biomass, suggesting that in *Chondrus crispus* the cellulose

fraction is low compared with *Ulva* biomass. Xylose was detected in trace amounts in the samples.

The total sugar content in *Chondrus crispus* biomass is 32.92%. In the extract, this percentage increases to 46.95%, while the residue contains a lower total percentage of 25.58%. In the case of *Chondrus crispus*, aqueous extraction increases sugar concentration, which is the opposite of *Ulva* sp. extraction. This effect is explained by the difference in cell wall polysaccharides solubility and composition between the two macroalgae with the content of soluble carrageenan being higher than ulvan.

CE uronic acid concentration (3.36 ± 0.10 % (m/m)), is lower than that of *UE* (26.9 ± 1.36 % (m/m)). Uronic acids are known to decrease pH and increase electrical conductivity[107], however these effects were not observed in the present study. Furthermore, there are conflicting reports that these compounds may interfere in plant development[107].

3.1.6. Phenolic content and antioxidant activity

The phenolic content in *Ulva* sp. water extract was found to be 5.03 mg of gallic acid equivalent per gram (mg GAe/g), while its antioxidant activity was measured at 3.60 mg of ascorbic acid equivalent per gram (mg AAe/g).

In the case of *Chondrus crispus* extract, the phenolic content was determined to be 2.89 mg GAe/g, and the antioxidant activity was measured at 1.87 mg AAe/g (Figure 19).

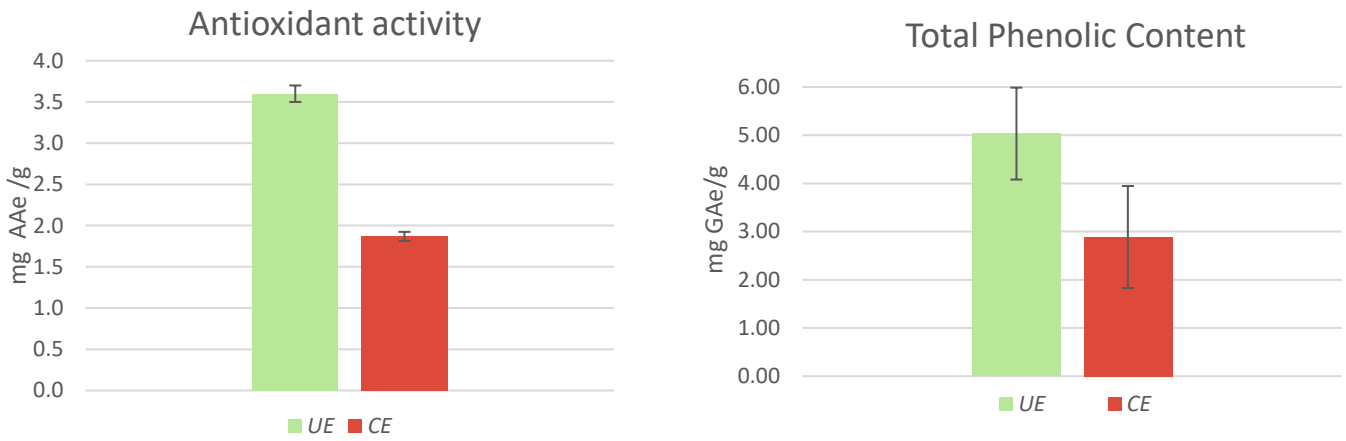

Figure 19- Antioxidant activity and Total Phenolic Content of Ulva sp. *and Chondrus crispus extracts*

Accounting for extraction yield, the *UE* attained 141 mg of GAe/100g of dried biomass, while the *CE* yielded 48 mg GAe/100g of dried biomass. Since polyphenols are recognized as potent antioxidants that play crucial roles in a plant's response to stress, with some even participating in growth regulation[122], achieving a robust extraction of polyphenols is a good indication of the effectiveness of the extract.

3.2. Seed germination results

The macroalgae water extracts were used in germination assays of cabbage and oat. After the designated time for germination, the Petri dishes were taken from the incubator (Figure 20), the seedlings carefully removed, the germination percentage was calculated (Figure 21) and the growth parameters measured.

Figure 20- - Example of the results of Oat Germination (left) and Cabbage Germination (right)

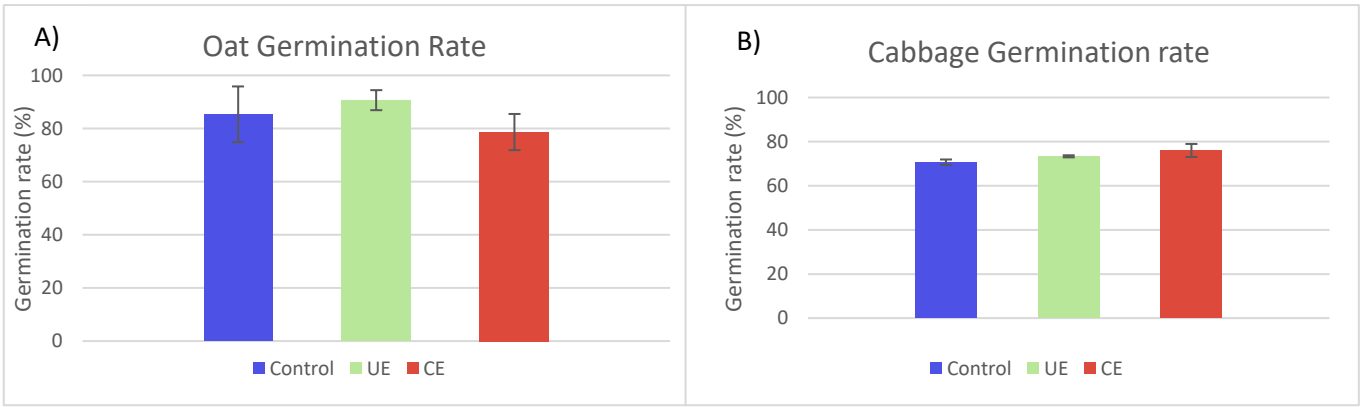

Figure 21- Oat germination rate (A) and Cabbage germination rate (B)

In the oat germination assay the *UE* appears to have had a positive effect and the CE a detrimental effect on germination rate although these effects were not significant.

In the cabbage germination assay a potential problem in seed quality was detected, many seeds had germinated but their growth had stunted immediately, in some cases the seed developed a short root, approximately 2 mm, and in other cases, a short leaf, approximately 2 mm. This phenomenon was observed randomly in all treatments, including the distilled water control, excluding the possibility of high electrical conductivity interfering with the germination. The slight acidic pH of the distilled water and extracts was another possibility, but it was ruled out because the same effect occurred in the juvenile growth assay where the seeds were placed in germination substrate and watered with tap water.

For consistency purpose, seeds which showed signs of germination, small leaves or small roots, were considered as germinated in germination rate calculations. In this context, *CE* had a slightly higher germination rate than the Control and *UE* treatment although this effect was not significant.

While the germination rate in the germination assays does not show significant differences between the treatments, the extracts do not exhibit negative effects on germination. Even though the extracts pH is slightly acidic and *CE* electrical conductivity is moderate.

The growth parameters included the measurement of the aerial part (from the base of the hypocotyl to the apical bud), radicular length and the fresh weight of the aerial and radicular parts was determined using an analytical scale. The growth parameters for the oat germination assay are illustrated in Figures 22 and 23.

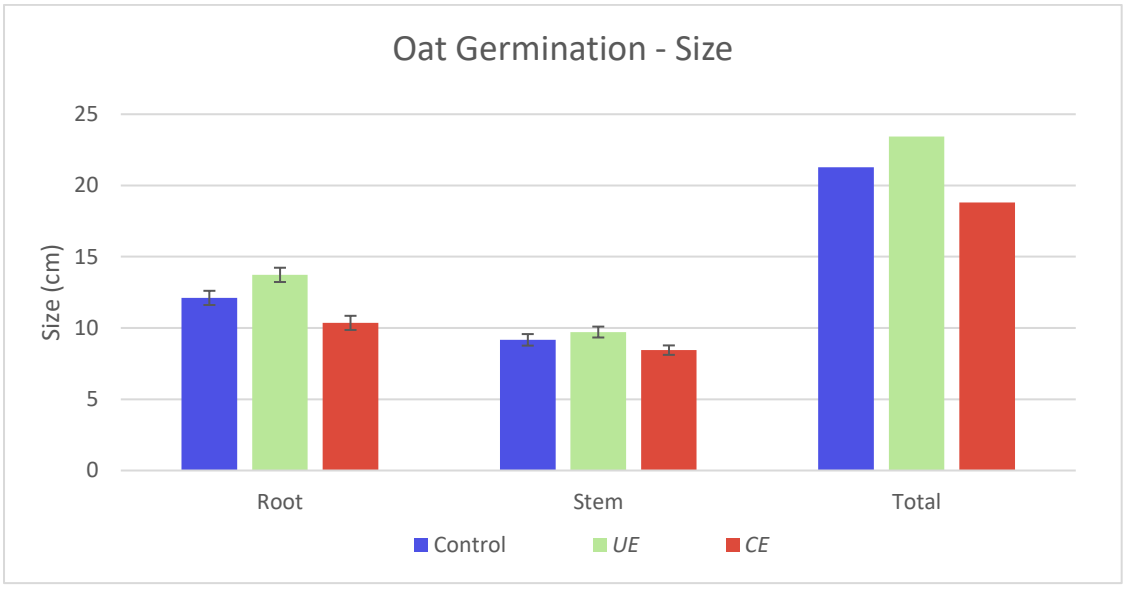

Figure 22- Oat germination size of root and stem

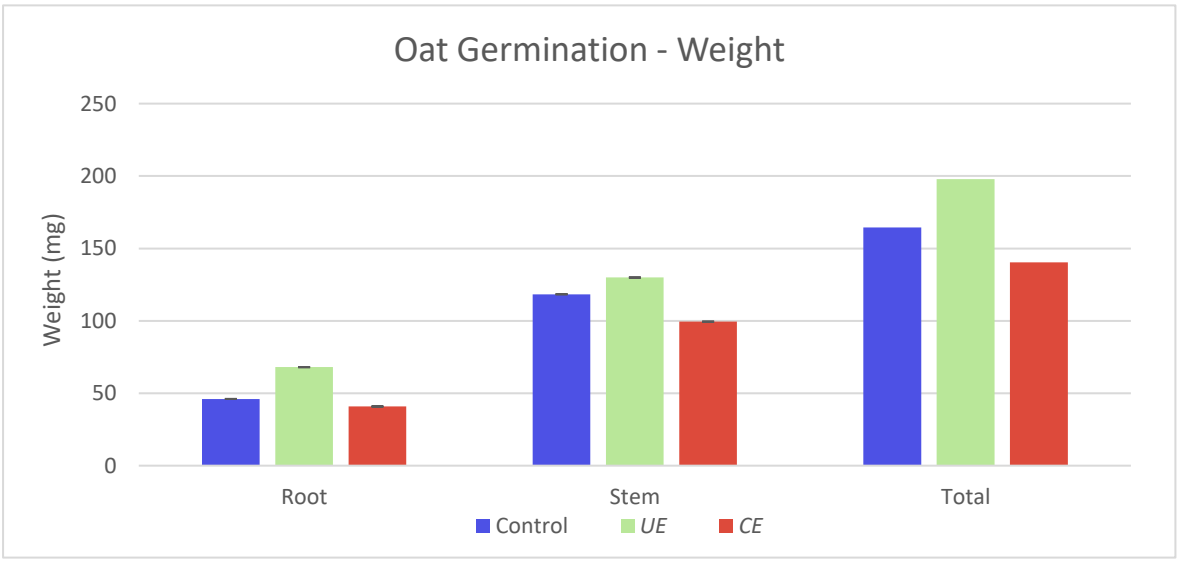

Figure 23- Oat germination weight of root and stem

The oat seedling growth results had different results for each macroalgae extract, *UE* appears to increase root and stem size although the results were not statistically significant($p>0.05$). Additionally, *UE* treatment increased significantly the root and total seedling weight($p<0.05$).

In contrast, *CE* was worse than *UE* in all growth parameters. Similarly, this extract was worse than the control in all growth parameters except stem size and radicular weight.

The growth parameters for the cabbage germination assay are illustrated in Figures 22 and 23. For analysis purpose, all cabbage seeds considered germinated had their growth parameters noted and statistically treated, this meant that all treatments had a population of very small or null measurements correspondent to the stunted growth seedlings, for example a seedling could have had 0 mg weight, 0 cm steam size and 0.2 cm root size. As a result of this population common to all treatments, there were no statistically significant differences between the treatments. However, UE and *CE* had an apparent positive effect on the successfully germinated seedlings, which resulted in the size and weight differences that can be observed in Figures 24 and 25.

There are reports in literature that identify carrageenan as the most beneficial macroalgae polysaccharide on *Brassica napus* and *Brassica oleracea* germination[95,107]. These findings align with the results of this assay, although UE had an apparent beneficial effect on cabbage germination, CE provided the best results.

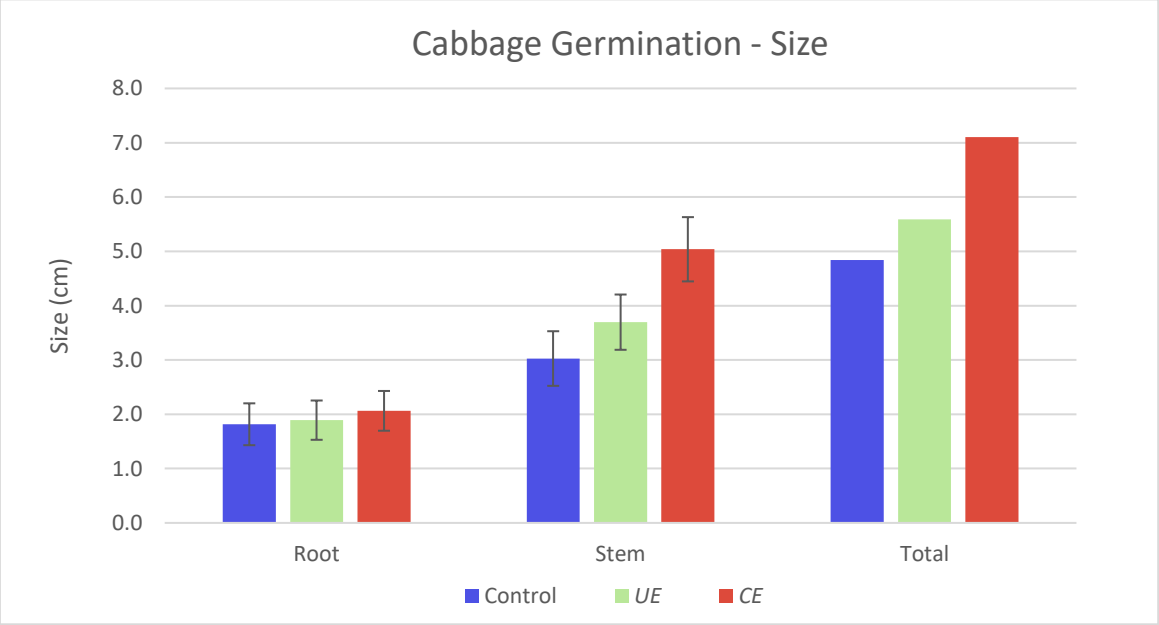

Figure 24- Cabbage germination size (cm) comparison

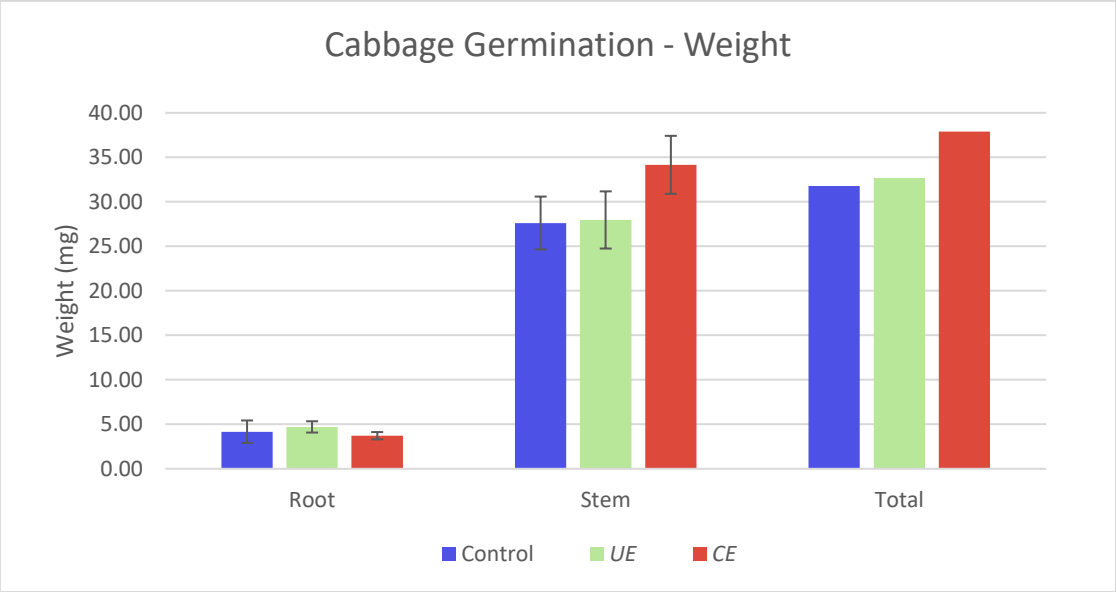

Figure 25- Cabbage germination weight (g) comparison

3.3. Juvenile growth treatments

Oat juvenile growth assay was conducted the foliar treatments applied after 7days (Figure 12) and the growth parameters measured at the 14th day of growth (Figures 26 and 27).

Figure 26- Oat juvenile grow after 14 days

Figure 27- Oat juvenile growth after 14 days

The growth parameters included the measurement of the aerial part (from the base of the hypocotyl to the apical bud) and radicular length. The fresh weight of the aerial and radicular parts was also determined. The growth parameters for the oat juvenile growth assay are illustrated in Figures 28 and 29.

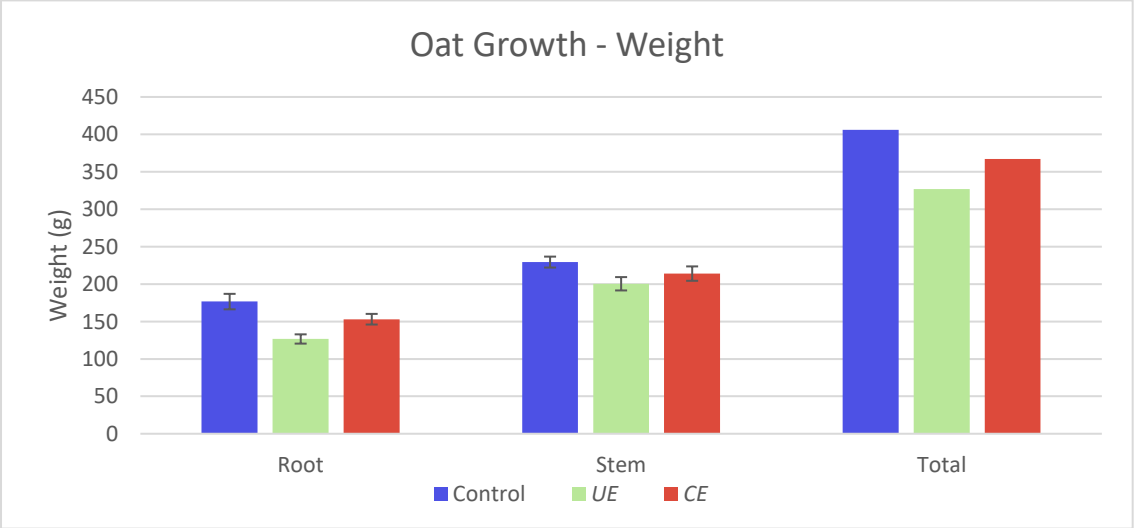

Figure 28- Oat growth weight (mg) comparison

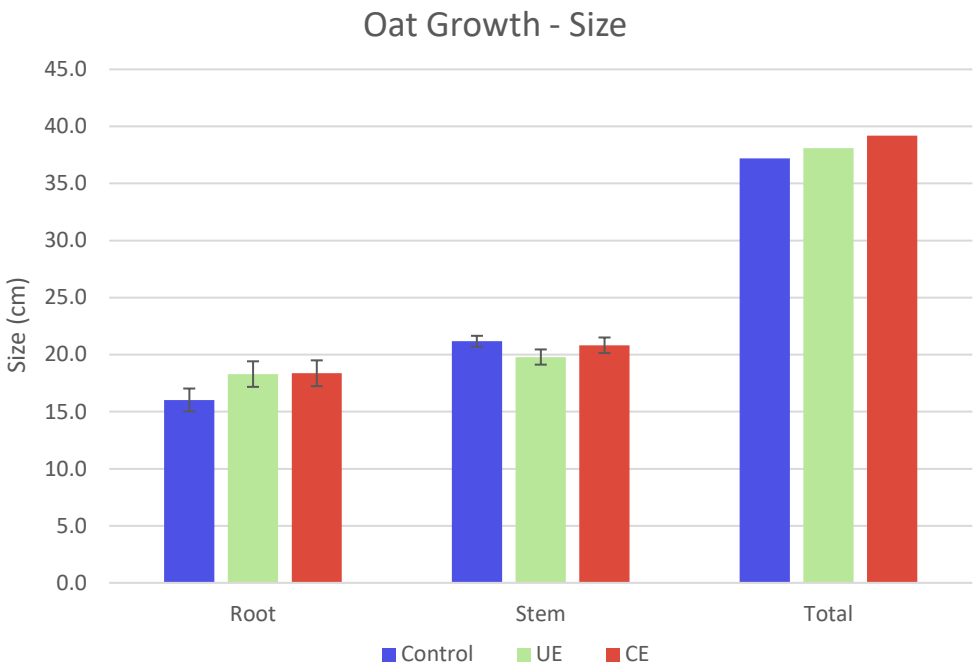

Figure 29- Oat growth in size (cm) comparison

The analysis of the oat juvenile growth results did not show significant differences between the treatments and the control. There was only one parameter which showed statistically significant differences which was the lower root weight of the treatment with *UE* vs the control($p<0.05$). Interestingly the medium root length of this treatment was higher than the other treatments, control with distilled water, and *CE* foliar application. This might be an indication that the extract promoted the formation of longer but less ramified roots, in drought stress conditions, the development of longer roots may help plants access humidity in higher soil depths[123].

The oat juvenile growth assay was conducted in ideal conditions, the plants grown in controlled humidity, and temperature. The juveniles had access to abundant light and were sown in quality soil with the different nutrients needed for plant development. In these conditions, the foliar application of the extracts did not have detrimental effects, suggesting that these treatments can be applied in future studies.

The cabbage juvenile growth assay suffered from the seed quality problems discussed in the cabbage germination assay, Figure 30 shows the germination tray after 14 days. This would

be the time were the different treatments would be applied. The seedlings show weak growth, evidenced by the inability to support their own weight. Additionally, the germination rate is randomly distributed in the germination tray. In the past, similar assays were conducted in the same laboratory with the same seed supplier and positive results, so the problem is likely seed batch specific.

In these conditions, it was not possible to conduct the foliar application of the treatment due to heterogeneous conditions across the germination tray and reduced number of individuals. Unfortunately, due to time restrictions, the assay could not be repeated.

Figure 30- Cabbage germination tray after 14 days

3.4. Plant biomass biochemical characterization

Pigments in plant biomass were identified by TLC, only the biomass correspondent to Oat juvenile growth had pigments in sufficient concentration for identification, which is expected as the germination assays were conducted in complete darkness, inhibiting the biosynthesis of substantial amounts of pigments. The chromatographic profile by TLC for oat juvenile growth assay is illustrated on Figure 31. The identified pigments and respective Rf are stated in Table 5, the pigments identified were chlorophyll a and b, photosynthetic

pigments, anthocyanins and carotene, which have antioxidant activity, and phaeophytin, a pigment involved in photosynthesis electron transfer.

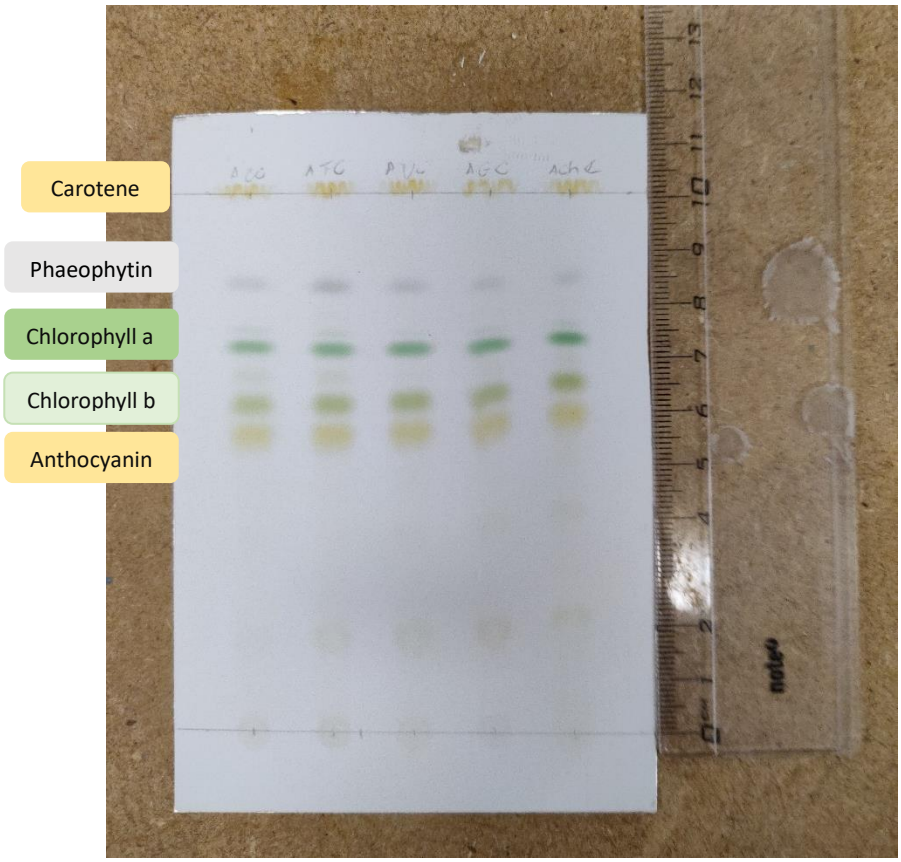

Figure 31- Chromatographic profile by Thin-layer chromatography (TLC) for oat juvenile growth biomass

Furthermore, chlorophyll a, chlorophyll b, carotenoids and anthocyanins quantification was carried out using UV-Vis spectroscopy. Figures 32 and 33 show the comparison in pigment content between the different treatments in the germination and juvenile growth assays.

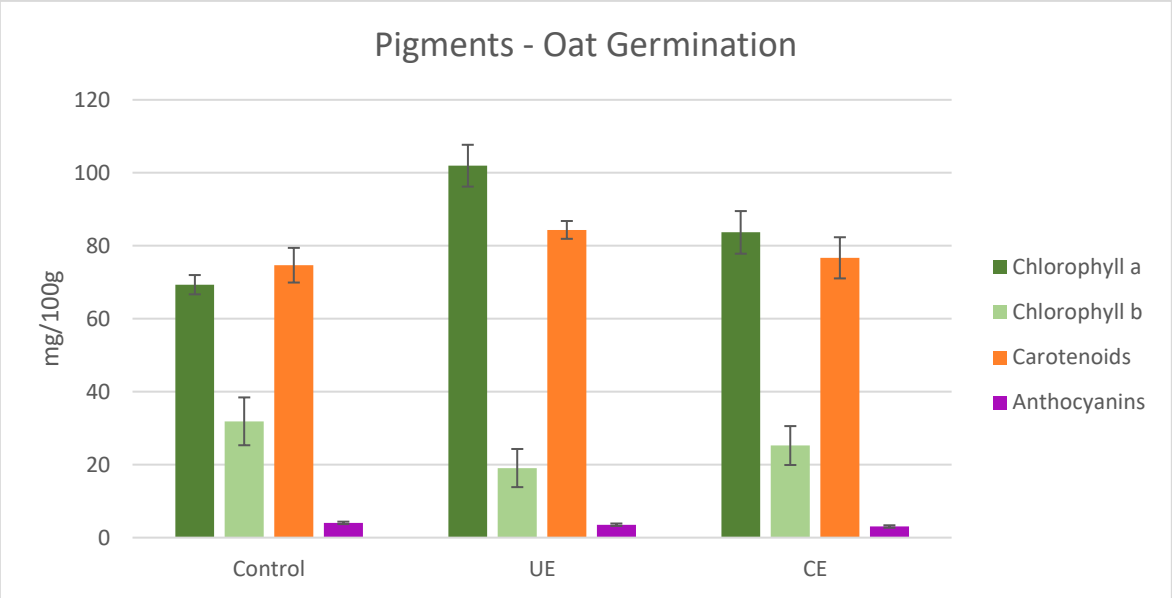

Figure 32- Pigments in oat germination biomass

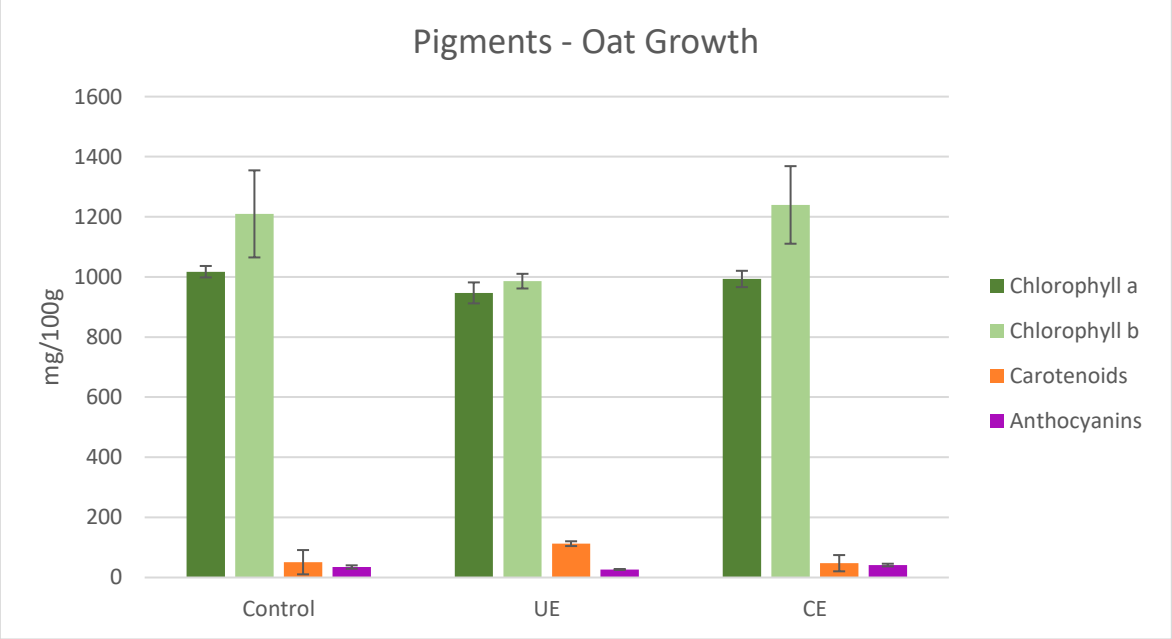

Figure 33- Pigments in oat growth biomass

In oat germination, the *UE*, which had produced the best growth results also had the higher Chlorophyll a content, the principal photosynthetic pigment in plants[110], indicating biostimulatory effects not only in seedling growth but also in potential photosynthetic activity.

Oat seedlings also had high relative carotenoids content when compared to oat juveniles, and in both assays carotenoids concentration is higher in *UE* treatments. In the case of oat growth, the difference could be related to lower chlorophyl concentrations which interfere with carotenoid calculation formula.

Oat carotenoids play an important role in plant protection against photooxidative stress, similarly, carotenoids have beneficial effects in human health, namely, antioxidant and anti-cancer activity[124].

Interestingly, carotenoid biosynthesis in cereal plants seeds is increased with germination, some researchers have recommended the induction of germination in cereal grains as a means of increasing carotenoid ingestion in human diets[125,126].

In the oat juvenile growth assay, the carotenoids relative content is lower. This is expected in plants in the juvenile phase. Additionally, the growing lights used in this assay don't emit damaging light in the UV spectrum like the sun, decreasing the need for photooxidative protection. Otherwise, the plants had a low Chl a/Chl b ratio; chlorophyll b is not essential for photosynthesis but plays crucial role in stabilizing the light-harvesting chlorophyll-binding proteins[127]. Under the abundant light conditions in this assay (16 light hours), photosynthesis is more productive than in normal growing conditions. In this context, the plants could have prioritized energy transfer and photosystem stability instead of photosynthesis.

Figure 34 shows the comparison in pigment content between the different treatments in the cabbage germination assay.

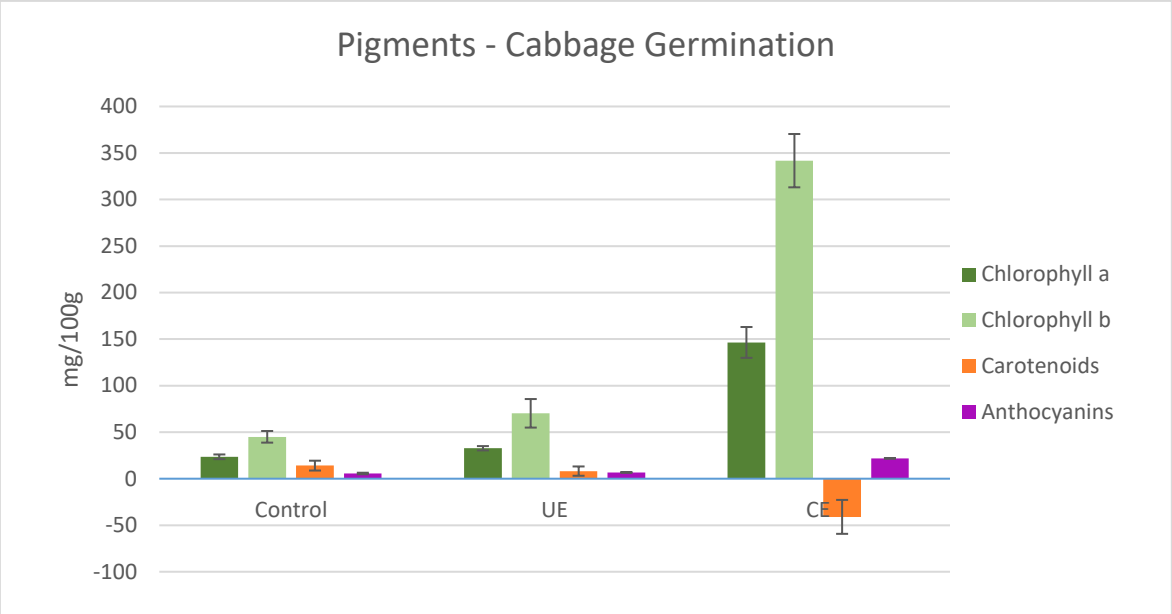

Figure 34- Pigments in Cabbage germination biomass

The *CE* significantly elevated chlorophyll a and b concentrations in cabbage seedling when compared to the treatment with the *UE* and the control. This extract was also the best in promoting growth in the seedlings. It seems that *CE* biostimulatory activity in cabbage seedlings has effects on multiple indicators of seedling development.

Compared with oat seeds, cabbage seeds are much smaller, as a consequence, cabbage seeds have significantly fewer reserve resources to use in the germination process. Compared with *UE*, *CE* had a higher sugar content. We hypothesize that in addition to the biostimulatory effect of *CE* carrageenans, the higher energy availability in the *CE* germination assay, allowed cabbage seeds to grow more efficiently.

Taking a more general approach, cabbage seedlings have significantly higher chlorophyll b concentrations than chlorophyll a. Chlorophyll b allows the absorption of a broader range of light wavelengths. High concentration of this compound is an adaptation to shade, as it permits photosynthesis in low light environments. Similarly, plants respond to low light environments by elongating their steams. This response called etiolation was observed in the germination assays and is correlated to chlorophyll b synthesis in cabbage seeds. It is important to note that cabbages are more suited to autumn, winter and spring conditions in which low light conditions are more common.

Carotenoid content was close to null in the cabbage germination assay. Negative values are the result of the calculation formula for carotenoids which considers absorbance in the chlorophyll range.

Anthocyanins have similar roles to carotenoids in plants, they are powerful antioxidants and provide photoprotection. However, the production of these pigments is more pronounced in other types of plants like some fruits and vegetables, as well as different cabbage varieties, like the red cabbage[111]. In this context, anthocyanins concentrations in all biomass samples were relatively low.

Carotenoids and anthocyanins have antioxidant activity which can be detected by the ABTS method. Additionally, compounds like ascorbic acid found in plant biomass, and macroalgae extract residues collected together with the seedlings, also contribute to antioxidant activity. The results for the ABTS assay conducted with biomass samples are shown in figure 35.

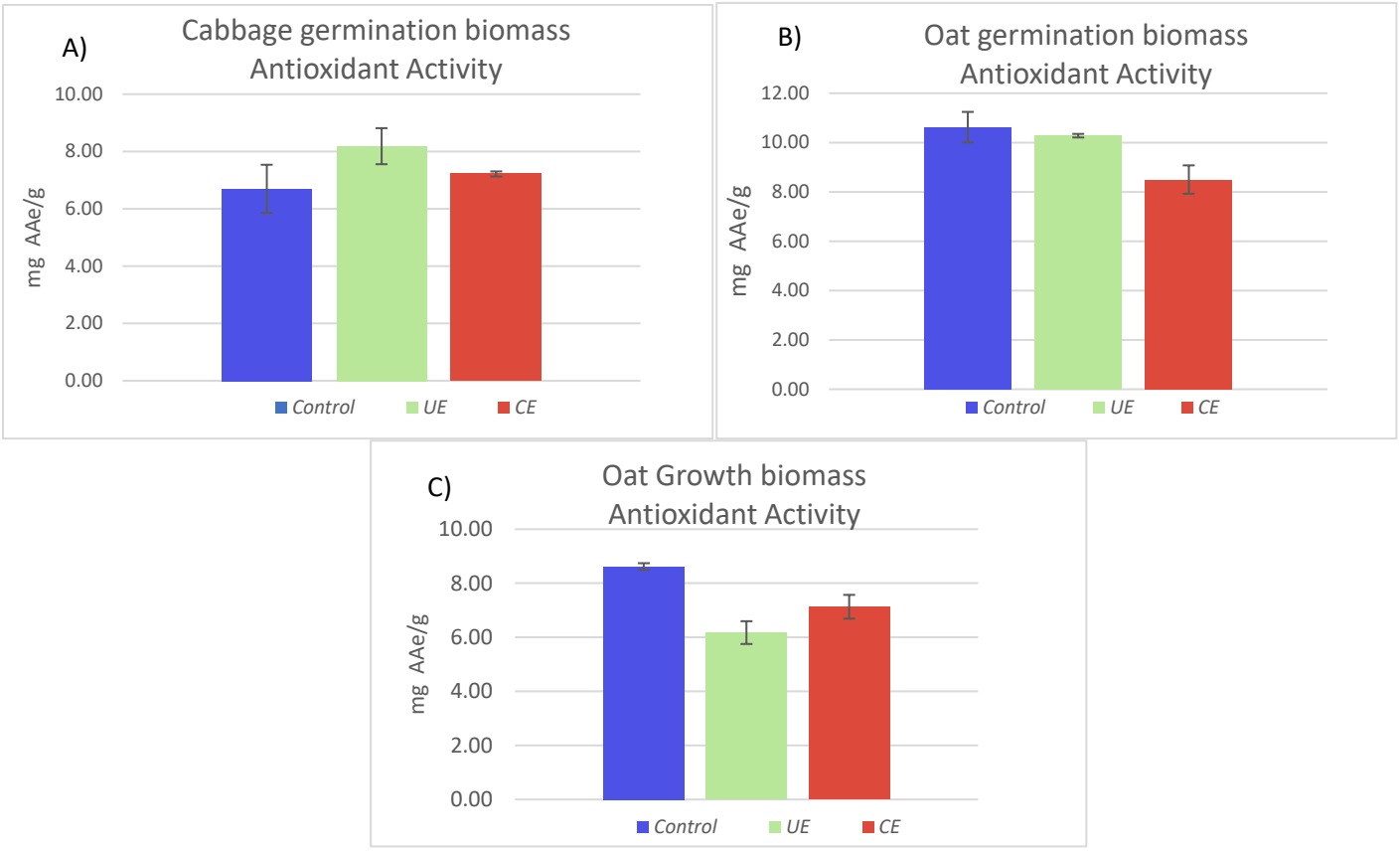

Figure 35- Plant biomass antioxidant activity, oat germination(A), cabbage germination (B) and oat growth (C)

In cabbage germination assay, *UE* treatment had higher antioxidant activity, although the treatment with *CE* had shown better biostimulatory activity. This may be explained by residues of extract present in the seedling biomass since *UE* showed higher antioxidant activity.

In oat germination assay the relatively lower antioxidant activity in *CE* treatment aligns with the lower overall growth observed in this treatment when compared to the control.

In the oat juvenile growth assay, the results are contrary to the expected, both *UE* and *CE* treatments resulted in lower antioxidant activity in the biomass. In published literature, there are numerous reports of increased antioxidant activity as a result of foliar application of macroalgae extracts[45,58,128]. In this case antioxidant activity was downregulated, it could be hypothesized that in ideal conditions (without stressors), macroalgae extracts downregulate stress response increasing focus on growth metabolism; however, we did not observe better growth in this assay, further research is needed to test this hypothesis.

Other, less common in this type of study, biochemical analysis were performed with the plant biomass collected, which included lipid and sugar analysis. In the case of these analysis, the experimental process used to separate the lipid and polysaccharide fractions was not successful, as a result of the diminute sample quantity, any biomass loss during the successive extractions and the noise to signal ratio in the chromatograms (Figure 36) produced unreliable lipid and sugar analysis results, nevertheless these can be consulted in the annex.

Furthermore, FTIR-ATR analysis were conducted with the germination and juvenile growth assays which can be seen in the figures 36, 37, and 38. As expected, the FTIR-ATR spectra did not produce significantly different results as the fundamental composition of each plant or seedling does not depend on the biostimulant used in each treatment. There are peaks related to O-H bridge and methyl groups in the 2850-2950 cm^{-1} range, and a peak at 1040 cm^{-1} correspondent C-O-C linkages, both related to organic compounds, as well as, bands related to proteins in the 1550-1650 cm^{-1} range.

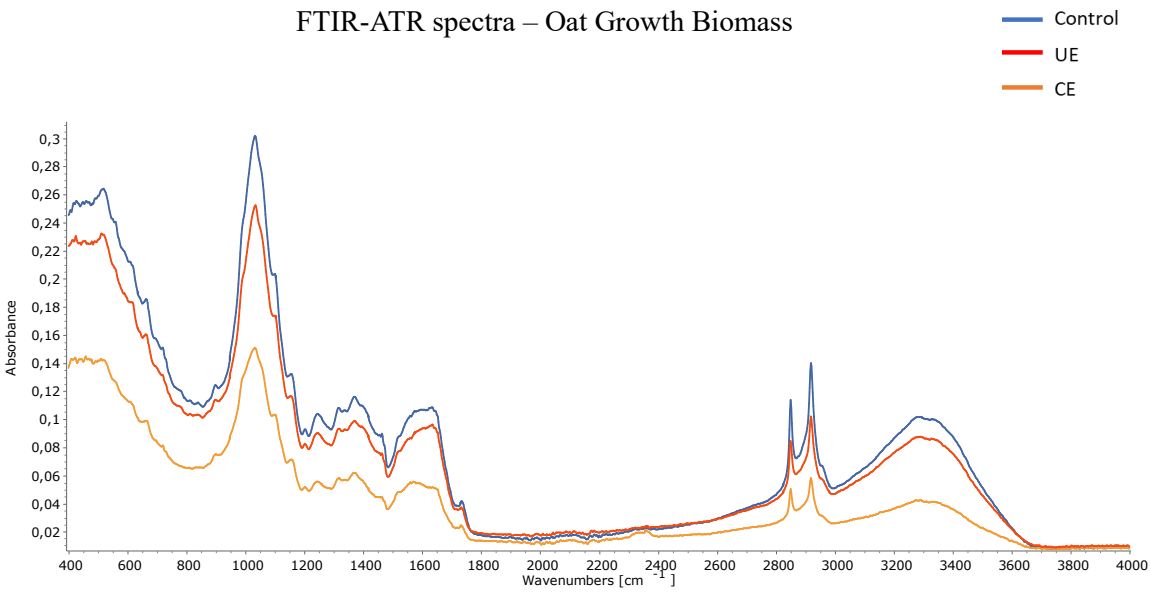

Figure 36- FTIR analysis of oat growth biomass

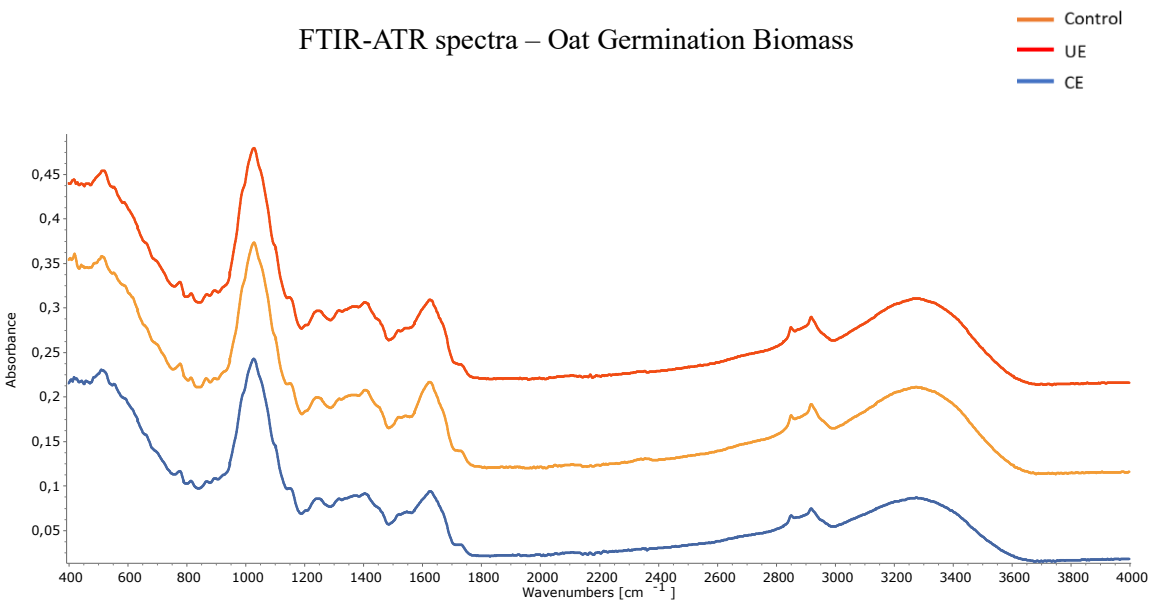

Figure 37- FTIR analysis of oat germination biomass

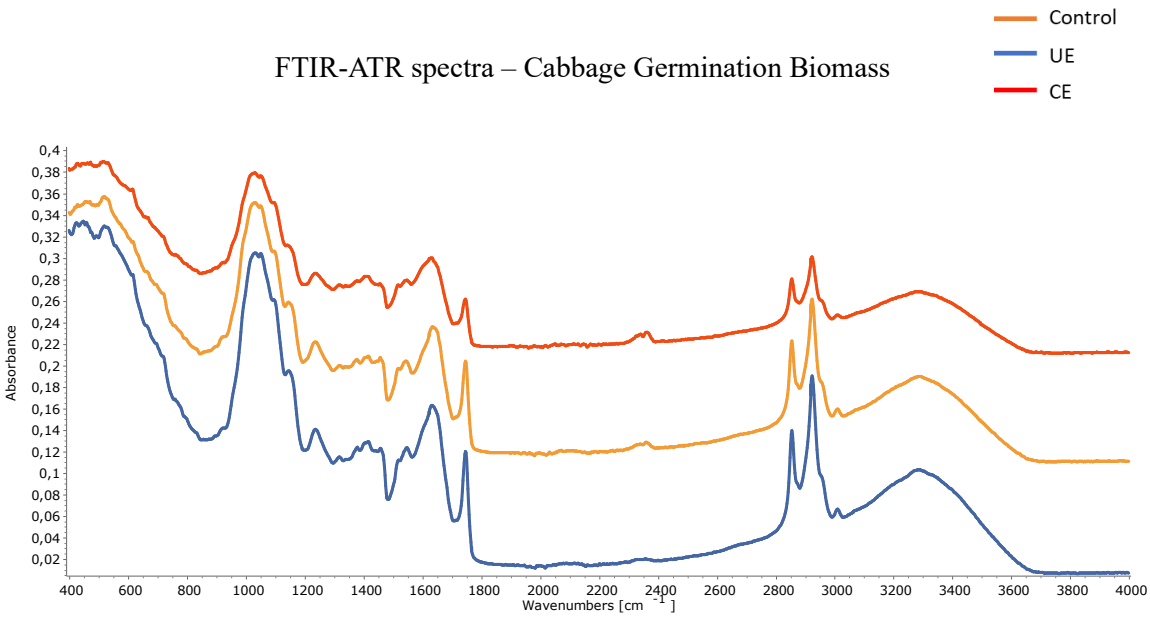

Figure 38- FTIR analysis of cabbage germination biomass

Interestingly, the elemental composition of the germination (Tables 5 and 6) assays with *UE* showed increased sulfur concentrations, this could be attributed to sulfur present in the extract but it is not clear why the same effect does not occur in the *CE* germination assays. Increased sulfur concentration can be a sign of increased biosynthesis of certain vitamins, defense molecules like glucosinates, and amino acids[129], although nitrogen content in the biomass was not higher. In contrast, there were no notable differences in oat growth biochemical analysis (Table 7).

Table 5- Oat germination biomass elemental composition

Oat Germination				
	Elemental composition in Percentage (%)			
Plant biomass	**C**	**H**	**N**	**S**
Control	38.611	5.727	3.606	0.371
UE	39.198	6.147	3.619	1.015
CE	38.477	5.991	3.803	0.327

Table 6- Cabbage germination biomass elemental composition

Cabbage Germination				
	Elemental composition in Percentage (%)			
Plant biomass	**C**	**H**	**N**	**S**
Control	41.223	6.682	3.964	1.057
Ulva sp.	39.998	6.432	3.739	2.000
Chondrus crispus	37.428	5.808	4.004	1.082

Table 7-Oat growth biomass elemental composition

Oat Growth				
	Elemental composition in Percentage (%)			
Plant biomass	**C**	**H**	**N**	**S**
Control	36.456	5.1385	3.008	0.2165
Ulva sp.	36.249	5.1935	3.023	- - -
Chondrus crispus	36.980	5.3780	3.005	0.2775

4. Conclusion and future research

In this work, water extracts of two macroalgae common in the Portuguese coast, *Ulva* sp. and *Chondrus crispus,* were prepared and biochemically analyzed. Substantial differences were found in the polysaccharide composition of each extract, *Chondrus crispus* extract was rich in carrageenan, and *Ulva* sp. polysaccharides were less soluble, ulvan being the main polysaccharide extracted. Both extracts had a rich mineral composition, *Ulva* sp. extract was higher in magnesium and manganese and *Chondrus crispus* extract higher in potassium and zinc. These differences help explain the different fertilization and biostimulatory activities of each extract.

The water extracts effectiveness as biostimulants was evaluated in germination and juvenile growth assays of two different crops of strategic importance, cabbage and oats.

Specifically, *Ulva* sp. extract was found to have biostimulatory effects on oat germination while *Chondrus crispus* extract was found to have detrimental effects in the same assay. In contrast, *Chondrus crispus* extract showed the best biostimulatory effects in the cabbage germination assay. Further research is needed to better understand the mechanisms responsible for the different effects observed in different crops.

Additionally, there were no beneficial effects observed following foliar application of the macroalgae extracts in oat juvenile plants growing in ideal conditions. Macroalgae biostimulants are particularly effective in non-ideal conditions, as they increase tolerance to biotic and abiotic stress.

In this context further research should evaluate the effectiveness of *Ulva* sp. and *Chondrus crispus* extracts in conditions similar with the ones of oat industrial cultivation. In Portugal, drought and heat stress are increasing in frequency. Therefore it is crucial to evaluate juvenile and adult growth under stress conditions to provide valuable insights to the prospects of commercial application of this type of biofertilizers.

In conclusion, the water macroalgae extracts produced in this investigation showed beneficial effects in seed germination. With previous extract selection, farmers can use macroalgae extracts to improve seedling vigor and potentially increase yields in an environmentally sustainable manner.

5.References

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6. Annex

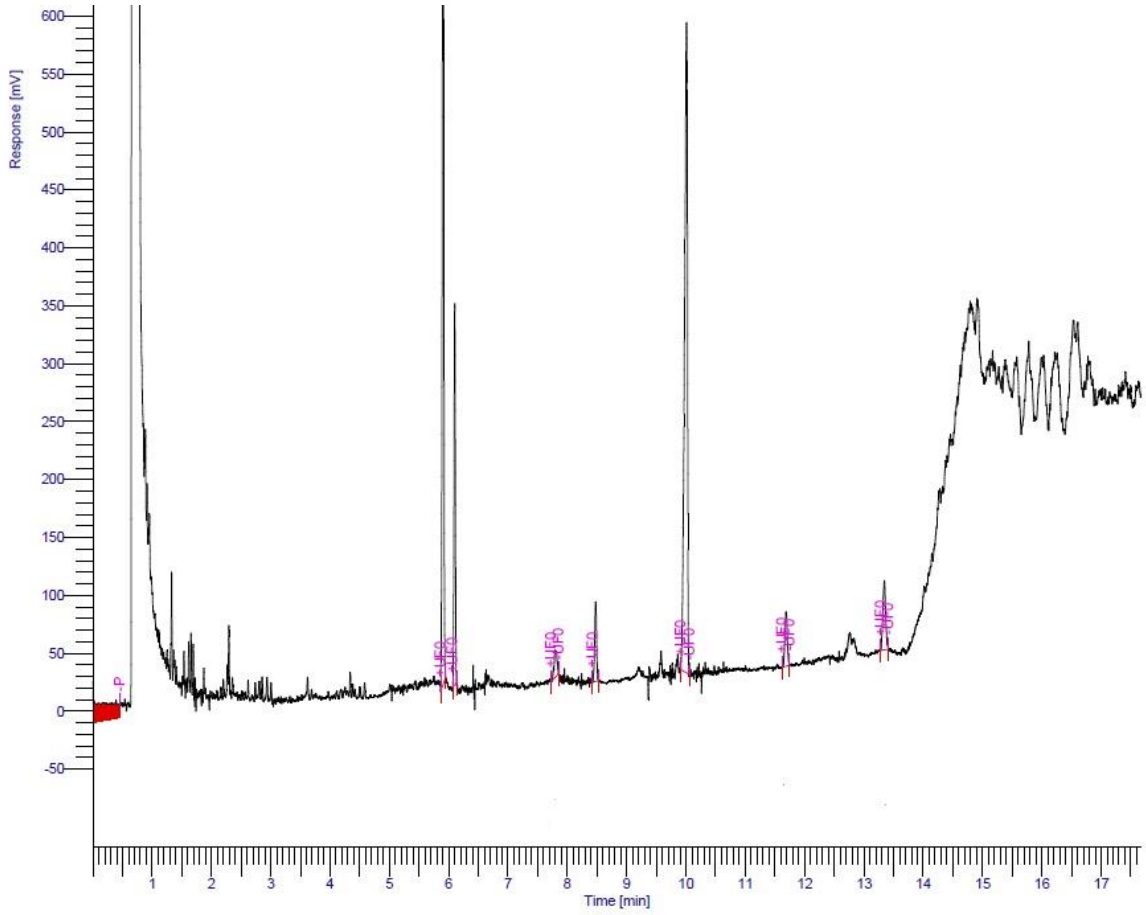

Figure 39- Example of lipid analysis chromatogram

Table 8- Oat germination biomass lipid analysis (m/m)

Oat Germination	C(10:0)	C(12:0)	C(14:0)	C(16:0)	C(16:1)	C(18:0)	C(18:1)	C(18:2)	C(18:3)	Total
Control	- - -	0.121	0.033	0.041	- - -	- - -	0.029	- - -	0.038	0.262
UE	- - -	0.481	0.211	0.046	- - -	- - -	0.040	- - -	0.083	0.861
CE	- - -	0.164	0.027	0.050	- - -	- - -	0.044	- - -	0.063	0.347

Table 9- Cabbage germination biomass lipid analysis (m/m)

Cabbage Germination	C(10:0)	C(12:0)	C(14:0)	C(16:0)	C(16:1)	C(18:0)	C(18:1)	C(18:2)	C(18:3)	C(22:0)	Total
Control	- - -	0.185	0.023	0.067	0.011	0.053	0.106	0.116	0.051	- - -	0.611
UE	- - -	0.100	- - -	0.074	0.029	0.052	0.168	0.137	0.082	- - -	0.642
CE	- - -	0.224	- - -	0.059	0.011	0.045	0.067	0.020	0.043	- - -	0.469

Table 10- Oat growth biomass lipid analysis (m/m)

Oat Growth	C(14:0)	C(16:0)	C(18:0)	C(18:2)	C(18:3)	C(20:1)	C(22:0)	Total
Control	0.299	0.351	0.262	0.655	0.488	0.077	0.163	2.296
UE	0.186	0.061	0.038	0.081	0.101	- - -	- - -	0.468
CE	0.084	0.062	0.043	0.049	0.106	- - -	- - -	0.344

Table 11- Oat growth biomass sugar analysis (m/m)

Oat Growth	Deoxyribose	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Control	0.673	0.092	0.178	0.235	0.245	0.441	0.519	0.114	0.277	5.435	8.239
UE	0.709	0.055	0.159	0.119	0.109	0.381	0.48	0.236	0.245	4.919	7.414
CE	0.856	0.083	0.200	0.228	0.087	0.163	0.776	0.157	0.273	6.318	9.141

Table 12- Oat germination biomass sugar analysis (m/m)

Oat Germination	Deoxyribose	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Control	0.561	0.034	0.190	0.344	1.046	0.514	2.013	4.749	0.287	8.187	17.925
EU	0.808	0.059	0.189	0.150	0.118	0.390	0.344	0.388	0.251	11.648	14.345
CE	0.639	0.073	0.173	0.238	0.057	0.360	0.330	0.309	0.284	9.693	12.157

Table 13- Cabbage germination biomass sugar analysis (m/m)

Cabbage germination	Deoxyribose	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Control	0.580	0.047	0.179	0.169	0.076	0.380	0.365	0.186	0.305	14.605	16.892
UE	0.493	0.060	0.193	0.210	0.154	0.412	0.286	0.230	0.286	15.588	17.769
CE	0.647	0.276	0.181	0.243	0.630	0.402	0.189	0.498	0.625	28.577	32.266