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Valorisation of macroalgal biomasses in eco-sustainable biotechnology

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1 Introduction to the application of algal biomass

The term “algae” has no formal taxonomic meaning, it is commonly used to indicate a polyphyletic group including photosynthetic organisms with various exceptions of colourless forms related to pigmented forms that can live in terrestrial and aqueous environments and grow in fresh, brackish, and saltwater. Nonetheless, they are found in almost every other environment on Earth, including “extreme” habitats, from rocks in the snow to algae living in lichen communities, to unicellular algae in deserts and urban environments (Barsanti and Gualtieri, 2022; Singh and Sharma, 2012). Therefore, algae can be defined as aquatic, oxygen-evolving photosynthetic autotrophs that are unicellular, colonial, constructed of filaments, or composed of simple pseudo-tissues (Guiry, 2012). Today's phylogeny and classification of higher taxa, including species, are more sophisticated than ever. The number of species and intraspecific names reported in AlgaeBase is approximately 168,332 (Guiry and Guiry, 2022), but estimates suggest that this number could exceed one million (Guiry, 2012). From unicellular species (between 2 and 40 μm) to multicellular algae (some of which can reach lengths of more than 70 m), the form and size are highly diverse with very different organisms (Ścieszka & Klewicka, 2019). This description includes several forms that may not be closely related, such as cyanobacteria, which are evolutionarily closer to bacteria than other algae.

The multicellular algae, macroalgae or seaweeds, are thallophytes (plants that lack roots, stems, and leaves) whose main photosynthetic pigment is chlorophyll *a* and lack a sterile covering of cells around

gametes. They function as primary producers in the food chain in various settings, generating organic matter from sunlight, carbon dioxide, nutrients, and water. They are categorized as red (*Rhodophyta*), green (*Chlorophyta*), and brown (*Phaeophyceae*) algae.

Currently, more than 200 species of macroalgae are harvested and/or cultivated for use directly or indirectly in biotechnology (as well as a culture medium for various plants and microorganisms), the pharmaceutical, cosmetic, textile, and food industries as texturizing agents with gelling and thickening properties (carrageenan, agar, alginate, and ulvan). Additionally, they are utilized as food and food supplements, soil conditioners, fertilizers (and/or foliar sprays), and some biomass is converted into bioplastics and other biomaterials. Additionally, eutrophication (nutrient depletion) and CO_2 carbonation can be reduced with seaweed farming. Some species, particularly those that are close to fisheries wastewater, can be utilized to clean wastewater, partially recover nutrients, and absorb heavy metals from industrial effluent. They are also used as fertilizer (and/or foliar spray), soil conditioner, feed and feed supplement, and some biomass is used to make bioplastics and biomaterials in general.

For more than three centuries, macroalgae have mostly been grown in Japan and China, primarily for human use. Algae cultivation began in Japan around 1700, when the first efforts to grow it inside of fish cages were attempted. However, it wasn't until early 1950 with an advancement in the cultivation of *Nori* algae that scientific farming

methods and a good culture technique were developed, leading to much more effective commercial production (*Porphyra*/*Pyropia* spp.). Seaweed can be cultivated in the marine environment on suspended lines, rafts, or nets. The most common cultivation technique is the long line due to its simplicity and low costs. Commonly used substrates for growing algae include ropes, rings, or nets. Depending on the species, seaweed is left to grow for months to year before of being harvested, and different groups of species belonging to green, brown, or red algae are cultivated using the long-line technique. These types of aquacultures farms may be set in the open sea, closed bays, lagoons, and estuaries. Another relevant cultivation technique, especially in warm temperate climates, is bottom culture, based on the insertion of thalli into a sandy bottom using different types of tools. Bottom planting is often applied to red algae species of the genera *Gracilaria* and *Kappaphycus* that are widely distributed all over the world, but most of the species are reported from tropical waters. Seaweeds can take up excess nutrients discarded by other species, such as fish or shrimp; for this reason, they are called bioextractive organisms. The integrated culture of fed aquaculture (fish and shrimp) with extractive aquaculture (seaweed and shellfish) is called 'Integrated Multi-Trophic Aquaculture' (IMTA). One of the most common methods for integrating seaweed mariculture is cultivating it close to the fish cages. The integration of fish and seaweed farming may help to solve the pollution issues since seaweeds can remove up to 90% of nutrient discharge from an intensive fish farm. More than

20 species, mainly brown algae, have been tested as potential biofilters of animal effluents in IMTA systems. Marine macroalgae farming suffers a few drawbacks, such as the possibility of introducing invasive species, grazing of weeds by fishes, fouling, and changes in nutrient composition at the cost of prolonged cultivation in the same location. It is necessary to consider these potential problems when planning to run a seaweed cultivation. In the past 30 years, the seaweed farming and production process have significantly improved, and today plays an important role in the worldwide aquatic industry (Cai et al., 2021). According to data of the Food and Agriculture Organization (FAO, 2012), the global seaweed production (both from aquaculture and wild harvesting) has increased from 120,000 tons to over 360,000 tons, from 2000 to 2019 (FAO, 2021). To date, 97% of the global seaweed output comes from artificial cultivation with Asia that accounting most of the entire world's production. In this continent the highest production involves in the highest technological skills and know-how. In this framework China ranks first in the world accounting for 57% of the global aquaculture production. The main seaweeds are the Japanese Kelp (*Saccharina japonica*), *Gracilaria* spp. and *Nori* algae (*Porphyra*/*Pyropia* spp.). The second in term of production is Indonesia, accounting nearly the 29% of the global output. In this country, the major cultivated algae on which the farms focus is *Eucheuma* spp. and *Kappaphycus* spp., almost always exported to other countries for the extraction of phycocolloids. Despite accounting only for 6% of the world production, South Korea

developed a seaweed culture industry processing many seaweed species, including brown, red, and green taxa; among them, *Saccharina* spp. and *Laminaria* spp. are the most cultured, followed by *Porphyra*/*Pyropia* spp. and *Undaria pinnatifida* (*Wakame*). Another important Asian country in term of production is Philippines, which reach 5% of the global market, mainly focused on *Kappaphycus alvarezii*. Japan accounts just for 2% of the global seaweed production, but with various seaweed from brown to red, especially with wide cultivation of *Porphyra*/*Pyropia* species. Very different is the situation in North America, in which the production extents no more than 2% of the world's seaweed, with 95% of which harvested from the wild. In South America, Chile is the main producer, accounting for 0.3% of the global production, and it mainly grows *Gracilaria* seaweeds, 99% of which coming from natural riverbeds. Europe accounts for 2% of global seaweed production. In Africa, Zanzibar accounts for 0.5% of the global aquaculture, mainly spiny *Eucheuma* spp., and Oceania accounts, that account less than 1% of the world and 99% come from cultured seaweed. It mainly produces miscellaneous of brown seaweeds, with several company focusing on *Asparagopsis* harvesting and cultivation, nowadays (Zanolla et al., 2022). In Europe, 97% of the seaweed is obtained from natural sources. Only since 2010, artificial cultivation has been experimenting in Europe, especially in Norway, Spain, France and Ireland. The focus is to shift to high quality species such as *Saccharina latissima* or *Pyropia*/*Porphyra* spp., but also to those with

high potential in the near future, such as *Aspargopsis* spp. and *Ulva* spp. (Lee et al., 2013; Araújo, 2020). The development of green algae resources is not completely explored. Novel compounds with biological activity have been discovered recently, and the interest and demand of algal compounds and other secondary chemicals is growing (Carpena et al., 2022; Michalak et al., 2022). Seaweeds contain a wide range of interesting bioactive compounds with nutritional value. Furthermore, algae can produce far more biomass than terrestrial plants and may be cultured successfully in fresh or seawater without the use of antibiotics or pesticides, which lead to an increase in consumer demand and economic interest over the last two decades. As previously described, seaweeds can be used as they are (or simply dried) as food, feed or food additive. In many cases the biomass must be processed before to be placed in as a finished product in the market. Different types of extracts can be classified based on; i) composition and chemical affinity; ii) the extraction techniques; and iv) intrinsic physical-chemical and biological peculiarities (Das, 2015; Kim, 2015; Olatunji, 2020). In the last decade, the extraction methods are often assisted by innovative additions, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized solvent extraction (PSE), and enzyme-assisted extraction (EAE). MAE is a relatively new extraction technique that combines microwave and traditional solvent extraction (Kumar, 2019). The use of microwaves generates heat directly in the matrix through the friction and collision between molecules. It has

been used to extract seaweed hydrocolloids and other derivatives from red and brown seaweeds to obtain high-quality seaweed hydrocolloids with less extraction time and solvent consumption (Le et al., 2019). Compared to the traditional polysaccharide extraction methods, MAE has a shorter extraction time, less solvent consumption and higher extraction rate (Delazar et al., 2012). UAE can be performed at low temperatures due to the vibrating- cavitation effect of the ultrasonic waves, which reduces heat loss at high temperatures and prevents the entry of bioactive substances. It is suitable for the extraction of heat-resistant compounds, it is simpler and faster than microwave extraction and it has great potential for large-scale production (Zhang et al., 2022). SFE is a process for extracting valuable substances using solvents at pressure and temperature above the critical points, which is environmentally friendly, inexpensive, widely available, non-flammable and timesaving. Carbon dioxide and water are the most common applied supercritical fluids. Algal hydrocolloid contains many bioactive substances that are sensitive to degradation at high temperatures. PSE is a relatively new automated technique that extracts target compounds at 200°C and 3000 psi using low-boiling solvents or solvent mixtures. Solubility, solvent diffusion and mass transfer rate were significantly increased by the PSE method, while solvent viscosity and surface tension were decreased. A wider range of solvents can be used for PSE extraction compared to SFE. However, PSE is not suitable for heat-resistant compounds that are sensitive to high temperature and high pressure and is not selective for

SFE. EAE uses specific enzymes to break down unwanted cell wall components, thereby releasing desired components. Compared with the traditional water extraction method, it has the advantage of high catalytic efficiency and preserves the original activity of the compound. It is necessary to select appropriate extraction technologies for different active substances. In particular, the combination technology has great potential to minimize the degradation of bioactive compounds caused by different extraction steps. Many bioactive substances from seaweed play an important role and have promising applications in functional foods, health care products, cosmetics, and medicine. However, more research is needed to improve modern extraction technologies to enlarge the industrial scale. In this sustainable view, others extraction method, developed for a different biomass, can be used to valorise algal biomass, which would be otherwise eliminated as waste. One such method is the Hydrothermal Carbonization (HTC). This process was explored more than a hundred years ago and in the last few years that has been reconsidered as an actual alternative for transforming wet biomass into a value-added product (Stemann et al. 2013; Zhu et al. 2015; Espro et al. 2021). The main product resulting from this technique is called hydrochar and has different physico-chemical characteristics due to several factors, including the starting biomass. Different applications of hydrochar were developed, including water retainers and soil amendment, but also finest uses as a low-cost adsorbent for contaminants, heavy metals and even pathogen from the water (Wang

et al. 2021). Hydrochar produced by algal biomass already has applications in adsorption of drug pollutants (Sachan and Das 2022), but an additional benefit relies in the use of waste biomasses as starting material that will be, therefore, converted into a valuable product other than displaying novel properties.

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2 Aim of thesis

The goal of the present doctoral thesis was to explore and improve some aspects of innovative processing methods of macroalgal biomass and their application in a sustainable way. The work is divided into two sections: i) innovative processing methods (§ 3.1; § 3.2; § 3.3); ii) uses of extracts and by-products (§ 4.1; § 4.2; § 4.3; § 4.4; § 4.5; § 4.6).

The species used have been chosen according to the goal to achieve and therefore for the final purpose a specific research topic. Biomass were obtained by harvesting natural populations growing in abundance in eutrophic systems, such as estuaries, lakes or ports, focusing on alien and/or invasive species. All the steps to obtain algal products were carried out with attention to the ecological and sustainable aspects of the processes.

Extracts and by-products were obtained through innovative processes in comparison with the conventional ones (§ 3.1). Furthermore, the use of some problematic biomass, such as those belonging to the genus *Sargassum*, was evaluated to obtain carbonaceous materials with high added value and low environmental impact (§ 3.2) and the residual liquid phase, considered as a waste, was wised as a germination activator (§ 3.3).

The diversity of products from seaweed, and their possible applications from pharmaceuticals to agriculture was explored. One of the most focused product categories was represented by polysaccharides from macroalgae (phycocolloids). Some of these have

been selected and tested for their antibiotic, antibiofilm, antisurfactate activities and antifouling properties (§ 4.1; § 4.2; § 4.3). Moreover, selected extracts were tested as biostimulants and plant growth regulators (PGRs), and the levels of PGRs were wised to implement the growth of crop and ornamental plants (§ 4.4; § 4.5; § 4.6).

Some of the presented findings were developed in collaboration with academic departments and/or industrial companies (§ 5).

3 Innovative processing methods

3.1 Conventional *vs.* Innovative Protocols for the Extraction of Polysaccharides from Macroalgae

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Conventional *vs.* Innovative Protocols for the Extraction of Polysaccharides from Macroalgae

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Abstract: Macroalgae are one of the most environmentally friendly resources, and their industrial by-products should also be sustainable. Algal polysaccharides represent valuable products, and the definition of new eco-sustainable extraction processes, ensuring a safe and high-quality product, is a new goal in the context of reducing the carbon footprint. The aim of the present work was to determine the influence of the extraction methodology on the properties and structure of the polysaccharides, comparing conventional and innovative microwave-assisted methods. We focused on extraction times, yield, chemical composition and, finally, biological activities of raw polymers from three macroalgal species of *Chlorophyta*, *Rhodophyta* and *Phaeophyceae*. The main objective was to design a sustainable process in terms of energy and time savings, with the aim of developing subsequent application at the industrial level. Extraction efficacy was likely dependent on the physico-chemical polysaccharide properties, while the use of the microwave did not affect their chemical structure. Obtained results indicate that the innovative method could be used as an alternative to the conventional one to achieve emulsifiers and bacterial antiadhesives for several applications. Natural populations of invasive algae were used rather than cultivated species in order to propose the valorization of unwanted biomasses, which are commonly treated as waste, converting them into a prized resource.

Keywords: alginate; antibiofilm assay; carragenans; emulsifying activity; DNA barcoding; Fourier transform infrared spectroscopy-ATR; gas chromatography with flame ionization detection; gel permeation chromatography; invasive macroalgal biomasses; ulvans

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1. Introduction

The polyphyletic group of marine macroalgae, due to their diversity, produces various chemical components. Among them, structural polysaccharides contained in their cell wall are the most valuable and are exploited commercially worldwide [1,2]. Nowadays, the world export of seaweeds amounts to more than USD 2.65 billion (more than 98 countries), of which USD 1.74 billion originate from seaweed hydrocolloids [3]. Macroalgae, and their derivate products, come from many commercial sectors, such as agriculture, feed, food, cosmetics and pharmaceutics, and many species are under investigation in the search for novel bioactive compounds [4–7]. Extraction of phycocolloids is one of the most profitable markets of macroalgae, which are used as thickeners, gelling agents and stabilizers. Thanks

to their structural and morphological versatility, they find applications in various industrial sectors not just as a food additive, but also in pharmaceutical and cosmetics products [8,9].

Polysaccharides useful for human affairs are mainly extracted from taxa of *Rhodophyta* and *Phaeophyceae*, but lately also *Chlorophyta*, which has entered fully into the market [10].

Species from *Rhodophyta* contain sulfated galactans, namely agars and carrageenans, a group of well-studied molecules with great variability [11,12]. Similarly, species of *Phaeophyceae* contain alginate, heteropolymers of guluronic and mannuronic acids, whose chemical characteristics vary by taxa, anatomical parts of thalli and extraction steps that result in acid and derivate salts [13]. Recently, other sulfated polysaccharides have been extracted from *Chlorophyta*, promising molecules used in nutraceutical, pharmaceutical and cosmetical applications and as antibiofilm agents [14].

Cultivated seaweeds are traditionally used to obtain phycocolloids, such as species of *Gracilariaceae* and *Gelidiaceae* for agar, species of *Solieriaceae* for carragenans and species of *Laminariales* for alginates [15]. However, the cultivation of macroalgae has a cost in terms of skilled personnel, equipment and facilities (including laboratories), which affects the retail price. Seaweed biomasses collected from anthropized coastal environments, where they thrive prosperously, obstructing human activities and, in part, also other organisms, might be dredged and used as an additional source of phycocolloids, given the pressing demand of the market [16]. Such populations have the added value of being able to resolve issues of disposal of unwanted biomasses, converting them into profitable products.

A variety of protocols have been proposed for obtaining raw polysaccharides, mainly by conventional chemical extractions [17]. In the last decade, novel methods have been tested, including extractions based on the use of enzymes, ultrasounds, microwaves and supercritical fluids [18]. Among them, microwave-assisted extraction has several advantages, namely a shorter processing time, a lower amount of solvent and a higher quality of derivate products [19,20].

The present research aimed to compare conventional and innovative polysaccharide extraction methods, focusing on microwave-assisted extraction, from different macroalgal species. Natural populations of the invasive *Ulva ohnoi* (*Chlorophyta*), *Agardhiella subulata* (*Rhodophyta*), both from the brackish lake of Ganzirri, in the Oriented Natural Reserve of Cape Peloro (Messina, Italy) and *Sargassum muticum* (*Phaeophyceae*), from the lagoon of Venice (Italy), were used to obtain ulvans, carrageenans and sodium alginate, respectively. Compared parameters were extraction times, polysaccharide yield and chemical composition. Obtained raw extracts were finally tested for their biological activities.

2. Materials and Methods

2.1. Sample Collection and DNA Barcoding

Samples of *Ulva ohnoi* (*Chlorophyta*) and *Agardhiella subulata* (*Rhodophyta*) were collected from the brackish lake of Ganzirri, in the Oriented Natural Reserve of Cape Peloro (Messina, Italy). Samples of *Sargassum muticum* (*Phaeophyceae*, *Ochrophyta*) were collected from the lagoon of Venice (Italy). Species names and *phylum* attributions are in accordance with algaebase.org [21].

After collection, samples were immediately washed with seawater to remove possible debris, adhered sand particles and associated organisms, transported to the laboratory in plastic bags at low temperature and washed with tap water to remove surface salt. Thalli were oven-dried at 40 °C for 48 h and stored in silica gel at room temperature until polysaccharide extractions.

From each fresh sample, a voucher was exsiccated on an herbarium sheet and a portion of thallus was subsampled, manually cleaned of epiphytes, dried in silica gel and stored at −20 °C for molecular identification.

Taxonomic identifications were performed through DNA barcoding methods, according to Miladi et al. [22] and Manghisi et al. [23]. Selected barcodes were COI-5P for *A. subulata* and *S. muticum* and *tuf* A for *U. ohnoi* [24]. Sequencing reactions were performed by an external company (Macrogen Europe, Amsterdam, The Netherlands). Forward and re-

verse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd., South Brisbane, QLD, Australia), and species attributions were performed by the identification engine in BOLD Systems (www.boldsystems.org, accessed on 6 March 2022).

Voucher specimens are housed in the Phycological Lab Herbarium (PhL, http://sweetgum.nybg.org/science/ih/herbarium-details/?irn=253162 (accessed on 8 March 2022). Collection information, voucher IDs, barcode identification numbers (BINs) and BOLD process IDs (PID) are listed in Table 1.

Table 1. List of the algal samples used in this study.

Phylum (Class)	Species	Collection Information	Voucher ID	BIN/PID
Chlorophyta (Ulvophyceae)	*Ulva ohnoi* M.Hiraoka et S.Shimada	Lake Ganzirri, Messina, Italy (38°15′28.8″ N 15°36′29.3″ E), 17 July 2020	PhL-APP028	GRAPP015-17
Rhodophyta (Florideophyceae)	*Agardhiella subulata* (C. Agardh) Kraft et M.J. Wynne	Lake Ganzirri, Messina, Italy (38°15′31.0″ N 15°36′49.5″ E), 30 June 2011	PhL-APP046	BOLD: AAC0053
Ochrophyta (Phaeophyceae)	*Sargassum muticum* (Yendo) Fensholt	Lagoon of Venice, Italy (45°25′42.6″ N 12°19′50.7″ E), 9 July 2020	PhL-APP031	BOLD: AAO5681

2.2. Extraction Protocols

Crude polysaccharides were obtained from each collected taxon with two different protocols implemented in parallel, a conventional protocol (CP) and an innovative protocol (IP), both modified from the literature [14,25–28], as detailed in Tables 2–4.

Table 2. Polysaccharide extraction protocols for *Ulva ohnoi*.

Conventional Protocol	Innovative Protocol
Incubate in distilled water at 70 °C for 3 h in convection oven.	Incubate in distilled water at 140 W for 10 min (70 °C) in microwave oven; repeat once, waiting until the mixture reaches 30 °C before the second incubation.
Use hot filtration with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

Table 3. Polysaccharide extraction protocols for *Agardhiella subulata*.

Conventional Protocol	Innovative Protocol
Soak in NaOH (2 M) at 70 °C for 2 h in convection oven.	
Filtrate with a cheese cloth and wash thalli in tap water to remove the solvent [(1)].	
Incubate in distilled water at 70 °C for 24 h in convection oven.	Incubate in distilled water at 140 W for 10 min (70 °C) in microwave oven; repeat thrice, waiting until the mixture reaches 30 °C before each incubation.
Hot filtrate with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

[(1)] Pretreated thalli can be dried at 40 °C for 48 h in convection oven and stored in silica gel at room temperature before subsequent extraction steps.

Table 4. Polysaccharide extraction protocols for *Sargassum muticum*.

Conventional Protocol	Innovative Protocol
Soak in HCl (0.01 M) at 25 °C for 24 h.	
Filtrate with a cheese cloth and wash thalli in tap water to remove the solvent [(1)].	
Incubate in 3% Na_2CO_3 at 70 °C for 24 h in convection oven.	Incubate in 3% Na_2CO_3 at 140 W for 10 min (70 °C) in microwave oven; repeat once, waiting until the mixture reaches 30 °C before the second incubation.
Use hot filtration with a cheese cloth to remove residual thalli.	
Precipitate in a volume of 94% ethanol at 25 °C for 24 h.	
Filtrate and centrifugate at 4000× *g*, 25 °C for 15 min.	
Dry precipitate at 40 °C for 48 h in convection oven.	

[(1)] Pretreated thalli can be dried at 40 °C for 48 h in convection oven and stored in silica gel at room temperature before subsequent extraction steps.

2.3. Chemical Characterization of Algal Extracts

The polysaccharide crude extracts obtained with CP and IP from each collected taxon were chemically analyzed. Determination of the main functional groups was performed by Fourier transform infrared (FTIR) spectroscopy, and molecular weights were determined by gel permeation chromatography (GPC). Monosaccharide composition was performed for polysaccharide crude extracts from *U. ohnoi* by gas chromatography with flame ionization detection (GC-FID) analysis.

2.3.1. Determination of the Functional Groups

The FTIR spectra of the dried extracted were determined with Nicolet iS 5 Thermo Scientific equipped with iD5 ATR (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The spectra were scanned at room temperature in the range of 4000–600 cm^{-1}, with a resolution of 4 cm^{-1} using a diamond crystal. For each sample, the measurement was performed in triplicate to obtain an average spectrum.

2.3.2. Determination of Molecular Weights

The molecular weight of the extracts was measured by gel permeation chromatography (Agilent GPC PL-GPC 220, Agilent Technologies, Inc., Santa Clara, CA, USA) with a refractive index detector (cell volume 8 µL, wavelength 890 nm).

The following conditions were used for each polysaccharide extract from *U. ohnoi* and *S. muticum*: 0.1 M sodium nitrate was used as a mobile phase at a flow rate of 1 mL min^{-1}, the temperature of the column was 30 °C and the injection volume of the aqueous solution containing the extract (3 mg mL^{-1}) was 100 µL [14].

For each polysaccharide extract from *A. subulata*, the same conditions were used with slight modifications, as follows: 0.05 M sodium nitrate as a mobile phase, column temperature set at 35 °C.

The calibration was performed using six pullulan standards with molecular weight in the range of 350–700 kDa.

2.3.3. Monosaccharide Composition

The assessment of the monosaccharide content in each polysaccharide extract from *U. ohnoi* was performed using gas chromatography with flame ionization detection (GC-FID) analysis (Agilent technologies HP 6890, Agilent Technologies, Inc., Santa Clara, CA, USA). Before the analysis, the extracts were subjected to acid methanolysis following the reported protocol [14]. In brief, 10 mg of each dried extract was soaked in 2 mL of 2 M HCl obtained by dilution of a commercial solution of 3 M HCl with anhydrous methanol (>99%). The methanolysis was carried out for 4 h at 100 °C. As the HCl concentration and the reaction time are crucial parameters affecting the release of the monosaccharides and their

stability, the methanolysis kinetics was investigated. For this purpose, two concentrations were used, 2 M HCl and 3 M HCl. Concentrations higher than 3 M were not used as they would result in monosaccharide degradation. The reaction was carried out for 4, 8, 12, 24, 48 and 72 h at 100 °C in triplicate, to find out optimal conditions to obtain the maximum release of polysaccharides with minimal degradation.

Following the methanolysis, the residual HCl was neutralized with pyridine (>99%). Sorbitol and methanol solution (0.1 mg mL^{-1}) were added, and the solvent was removed with rotavapor. The dried product was dissolved in a mixture made of 150 mL of pyridine, 150 mL of bis (trimethylsilyl)-trifluoroacetamide and 50 mL of chlorotrimethylsilane and kept for 30 min at 80 °C under stirring.

The quantitative determination of monosaccharides was performed by gas chromatography with FID detector (GC-FID). Detector temperature was set at 290 °C, injector temperature at 250 °C, and the program was set as follows: raise to 100 °C, from 100 °C to 180 °C at 4 °C min^{-1} and from 180 °C to 290 °C at 8 °C min^{-1}.

The external calibration was conducted using Rha, Xyl, GlcA, Glc and IdoA. All standards had purity >99%.

2.4. Microbiological Tests

2.4.1. Antibacterial Activity

The antibacterial activity of crude extracts against *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 was evaluated by the standard disk diffusion method (Kirby Bauer test), as accepted by the Clinical and Laboratory Standards Institute (CLSI) [29].

Pseudomonas aeruginosa and *S. aureus* were grown overnight on lysogeny agar (LA) or tryptic soy agar (TSA) plates, respectively, and incubated at 37 °C for 24 h, and colonies from each strain were suspended in 3 mL of 0.9% NaCl solution ($OD_{600\,nm}$ = 0.1). Aliquots of each suspension (100 µL) were inoculated onto plates of Muller Hinton agar (Oxoid Holdings ltd, Hampshire, UK) in triplicate.

Each algal extract (20 mg) was dissolved in 1000 µL of sterile distilled water, and 20 µL of each solution was applied to sterile filter paper disks (6 mm in diameter, Oxoid Holdings Ltd., Hampshire, UK). After evaporation, the disks, each containing 400 µg of extract, were placed onto inoculated plates. Plates were incubated overnight at 37 °C. The diameter of inhibition zones was measured, and means and standard deviations (SD) (n = 3) were calculated.

2.4.2. Antibiofilm Assay

The antibiofilm activity of algal extracts against *P. aeruginosa* and *S. aureus* was carried out in 96-well polystyrene microplates (Falcon®, Fisher Scientific, Milan, Italy), as previously reported by [30]. Overnight cultures (180 µL) of *P. aeruginosa* grown in Luria–Bertani (LB) or *S. aureus* in tryptic soy broth (TSB) (6 replicates) ($OD_{600\,nm}$ = 0.1 equivalent to 1.5×10^8 bacteria m L^{-1}) were poured in the microwells, and each crude polysaccharide extract (20 µL), diluted in phosphate-buffered saline (PBS, Sigma Aldrich, St. Louis, MI, USA) at 400 µg mL^{-1} or 20 µL of PBS used as a control, were added to each well. Microplates were incubated at 37 °C for 48 h (for *P. aeruginosa*) or 24 h (for *S. aureus*), without shaking. Nonadherent bacteria were removed by washing the samples 5 times with distilled water. Biofilms were stained with 0.1% (w/v) crystal violet solution for 20 min. Excess stain was removed by aspiration, and then the plates were washed 5 times and air-dried for 45 min. The stained biofilms were solubilized with absolute ethanol, and the biofilm mass was spectrophotometrically determined ($OD_{585\,nm}$) by the level of the crystal violet present in the de-staining solution, using a microtiter plate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA).

The reduction in biofilm formation of each strain was expressed as antibiofilm activity (%) by applying the following formula:

$$(OD_{control} - OD_{sample}/OD_{control}) \times 100.$$

2.4.3. Emulsifying Activity

The emulsifying activity of crude algal extracts was evaluated according to the method described by Mata et al. [31]. Each crude lyophilized extract was dissolved in 2 mL of distilled water (0.05%, *w*/*v*), mixed with an equal volume of kerosene in a glass tube (5 cm high and 1 cm in diameter) and stirred at high speed in the vortex for 2 min. The emulsion and aqueous layers were measured after 24 h, and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture, and multiplying by 100 [32].

2.4.4. Coating Assay for Polystyrene Surfaces

The ability of the extracts to modify the hydrophobic surface properties was investigated by the coating assay. Briefly, a volume of 20 µL of each extract diluted in PBS was transferred to the center of a 24-well polystyrene microtiter plate (Falcon no. 353047) [33]. To allow the complete evaporation of the solutions the plates, were incubated for 30 min at 37 °C. Aliquots (1 mL) of diluted overnight bacterial culture (containing 10^5 CFU mL^{-1}) in LB for *P. aeruginosa* and TSB for *S. aureus* were poured into each well of microplate. After incubation at 37 °C for 18 h in static conditions, the wells were emptied gently, washed with distilled water and stained with 1 mL of 0.1% crystal violet solution. To remove the excess crystal violet, wells were rinsed with distilled water and air-dried.

2.4.5. Statistical Analysis

Statistical analysis was performed using two-way ANOVA. Differences were considered statistically significant at a *p*-value of <0.01 or 0.05.

3. Results and Discussion

Polysaccharide yields and processing time of both the conventional protocol (CP) and innovative protocol (IP) for the extractions of *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* are shown in Table 5.

Table 5. Polysaccharide yields and processing time of extractions from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* for both conventional protocol (CP) and innovative protocol (IP). The yields are expressed as the average ± SD (*n* = 3).

Extract	Yield (g)	Time (h)
Ulva ohnoi CP	4.52 ± 0.72	3
Ulva ohnoi IP	13.20 ± 0.88	0.33
Sargassum muticum CP	38.38 ± 0.47	24
Sargassum muticum IP	33.67 ± 1.11	0.33
Agardhiella subulata CP	24.13 ± 0.25	24
Agardhiella subulata IP	28.50 ± 0.41	0.66

Innovative protocol extractions gave higher yields from *U. ohnoi* and *A. subulata*, but lower values from *S. muticum*, showing that the extraction efficacy is likely dependent on polysaccharide type. However, with the notable situation of *U. ohnoi* polysaccharides, whose yield almost tripled in IP extraction, the yields of both *A. subulata* and *S. muticum* polymers were comparable between the two tested methods.

Results of chemical analyses and microbiological tests are presented below.

3.1. Characterization of Extracted Polysaccharides

3.1.1. Determination of the Functional Groups

The chemical structure of the extracted polysaccharides is directly related to their chemical and biological properties, and any change could cause either a favorable or an adverse variation. As a consequence, the effects of the extraction procedure were evaluated by FTIR-ATR spectroscopy by verifying the presence of characteristic peaks of expected polysaccharide types in each extract.

The FTIR-ATR spectra are presented in Figure 1.

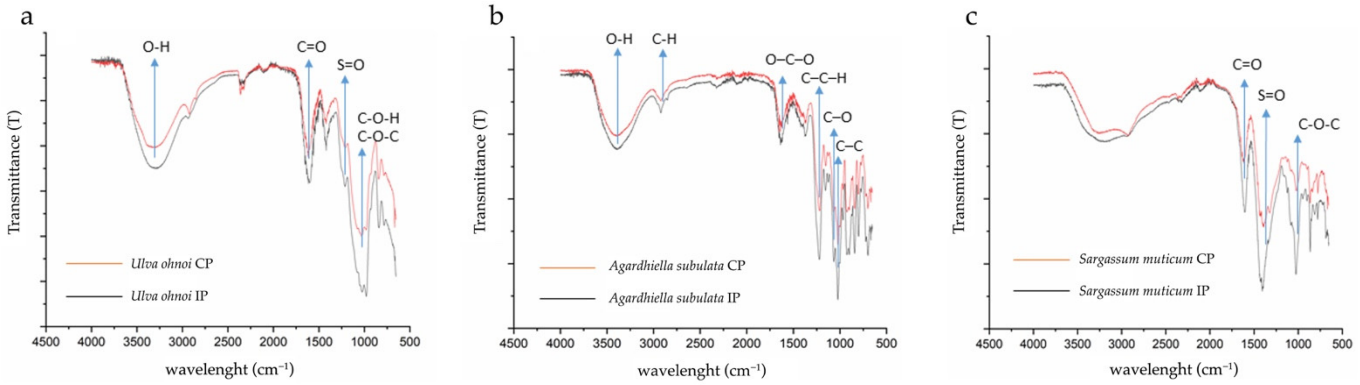

Figure 1. FTIR-ATR spectra of polysaccharide extracts (**a**) from *Ulva ohnoi*; (**b**) from *Agardhiella subulata*; (**c**) from *Sargassum muticum*. The black curves refer to conventional protocol (CP), while red curves refer to innovative protocol (IP).

Both spectra of *U. ohnoi* extracts (Figure 1a) confirm the structure of ulvans, in agreement with data reported in the literature [14,34]. The peak at 1653 cm^{-1} refers to the asymmetric stretching of the C=O while the band at 1248 cm^{-1} is related to the S=O. The peaks at 846 cm^{-1} and 800 cm^{-1} are related to sugar rings. The region around 1200–1000 cm^{-1} includes stretching vibration of C-OH groups and the C-O-C vibrations. The peak at 1050 cm^{-1} is related to the stretching of the two main monosaccharides in *U. ohnoi*, rhamnose (Rha) and guluronic acid (GlcA).

In the spectra related to *Agardhiella subulata* extracts, the peaks confirm the presence of carrageenan (Figure 1b). In fact, as reported [14], the peak at 1410 cm^{-1} is ascribed to the sulfate ester while that at 1020 cm^{-1} to the glycosidic bridge C-O-C. In the ranges of 920–940 cm^{-1} and 830–850 cm^{-1}, the peaks correspond to 3,6 anhydrogalactose and galactose-4-sulfate, respectively.

For both extracts of *S. muticum*, the presence of alginate is confirmed (Figure 1c). The large band centered around 3400 cm^{-1} is ascribed to O-H stretching vibrations while at 2900 cm^{-1} the C-H stretching vibrations are recognizable, and the asymmetric stretching of carboxylate O-C-O vibration is visible at 1620 cm^{-1}, as reported in the literature [35]. The peak at 1420 cm^{-1} might be assigned to C-OH deformation vibration with the contribution of O-C-O symmetric stretching vibration of the carboxylate group. Minor bands at 1300, 1120 and 1090 cm^{-1} are related to the C-C-H and O-C-H deformation, C-O stretching and C-O and C-C stretching vibrations, respectively, in the sugar ring. The peak at 950 cm^{-1} can be appointed to the C-O stretching vibration of uronic acid residues, and that at 880 cm^{-1} can be assigned to the deformation vibration of the C1-H in β-mannuronic acid residues. The band at 815 cm^{-1} is also ascribed to the mannuronic acid residues [36,37].

For all tested species, both CP and IP spectrum pairs overlap without substantial differences in the position and number of peaks. However, hypochromic effects were observed at a magnitude that varied with the functional group involved in all IP extracts. The obtained FTIR-ATR spectra suggest that the use of the microwave, at the frequency, duration and number of cycles tested, did not affect the chemical structure of the polysaccharide extracted.

3.1.2. Determination of Molecular Weights

The molecular weight (MW) is a key parameter as it influences the chemical, biological and physical properties of the extracted polysaccharides. In addition, it is highly sensitive to the extraction procedures and its evaluation is fundamental to understanding the occurrence of the degradation process during extraction.

The MWs of the polysaccharides extracted with both the CP and IP are presented in Table 6.

Table 6. Molecular weight (MW) and refractive index (RI) of polysaccharides extracted by both conventional (CP) and innovative (IP) protocols. The measurements were performed in triplicate, and the data are expressed as average (SD up to 10%).

Extract	MW_1 (kDa)	RI_1 (mV)	MW_2 (kDa)	RI_2 (mV)	MW_3 (kDa)	RI_3 (mV)
Ulva ohnoi CP	56	8	-	-	-	-
Ulva ohnoi IP	53	6	-	-	-	-
Agardhiella subulata CP	489	41	69	15	-	-
Agardhiella subulata IP	320	27	35	11	10	6
Sargassum muticum CP	157	12	21	7	-	-
Sargassum muticum IP	83	9	-	-	-	-

The acquired values show that an effect of the microwave during the extraction procedure could occur. In the case of *U. ohnoi* specimens, a monomodal distribution centered at 56 kDa (CP) and 53 kDa (IP) was observed.

Conversely, in the case of carrageenan, the CP-derived *A. subulata* extract, a bimodal distribution was observed at the major peak corresponding to MW 400–560 kDa, while the minor one represents a fragment between 55 and 110 kDa. In the IP-derived *A. subulata* extract, the main peak has a lower molecular weight, between 248 and 389 kDa, while the minor peak is between 20 and 50 kDa. In addition, a small peak is present in the range from 5 to 16 kDa, suggesting the presence of fragments.

In the case of alginate, in the *S. muticum* CP extract, a bimodal distribution was observed with the major peak corresponding to MW 110–205 kDa, while the minor one represents fragments between 10 and 35 kDa. In contrast, in the *S. muticum* IP extract, a monomodal distribution was observed with the peak shifting towards a lower molecular weight with a narrow distribution in the range of 60–95 kDa. The data presented in Table 6 suggest that a reduction in the molecular weight takes place when microwaves are applied during extraction.

3.1.3. Monosaccharide Composition

The composition of polysaccharides extracted by both conventional (CP) and innovative (IP) protocols is presented in Table 7. The data display that the whole content of monosaccharides obtained from *U. ohnoi* via methanolysis (2 M HCl and 4 h) was approximately 25%, which is in accordance, and in some cases higher, than values reported in the literature [38]. Rha and GlcA were the main residues, reaching 30% and 45%, respectively, in the CP extract, and 27% and 48%, respectively, in the IP extract.

The data in Table 8 confirm Rha and GlcA as the main monosaccharides, with a content of 28% and 52%, respectively, in the CP extract, and of 29% and 56%, respectively, in the IP extract.

Rha and GlcA represent the main monosaccharides; however, when increasing the HCl concentration from 2 M to 3 M, the total number of released monosaccharides increased by 33% and 42% with the CP and IP, respectively. This finding reflects the trend reported in previously published work, in which the same experimental conditions were used for the release of monosaccharide units from ulvan [38].

Monosaccharide content released by methanolysis over time at different acid concentrations, expressed as percentages of the dried weight of the extracts, is presented in Figure 2.

Table 7. Monosaccharide composition of the polysaccharides extracted by both conventional (CP) and innovative (IP) protocols from *Ulva ohnoi* (expressed as percentage of dry weight of the extract) after acid methanolysis using 2 M HCl/MeOH, for 4 h at 100 °C. The data are expressed as the average ± SD (n = 3).

		***Ulva ohnoi* CP**	***Ulva ohnoi* IP**
Monosaccharide	Rha	7.5 ± 0.2	7.1 ± 0.3
	GlcA	11.3 ± 0.7	12.7 ± 0.9
	IdoA	2.2 ± 0.1	1.8 ± 0.1
	Glc	1.9 ± 0.1	1.5 ± 0.1
	Xyl	2.1 ± 0.1	2.9 ± 0.2
Disaccharide	Rha-GlcA	2.3 ± 0.1	1.4 ± 0.4
	Total	27.3 ± 1.5	27.4 ± 2

Table 8. Monosaccharide composition of the polysaccharides extracted by both conventional (CP) and innovative (IP) protocols from *Ulva ohnoi* (expressed as percentage of dry weight of the extract) after acid methanolysis using 3 M HCl/MeOH, for 4 h at 100 °C. The data are expressed as the average ± SD (n = 3).

		***Ulva ohnoi* CP**	***Ulva ohnoi* IP**
Monosaccharide	Rha	10.4 ± 0.8	11.3 ± 0.5
	GlcA	19.1 ± 1.1	21.7 ± 1.1
	IdoA	2.7 ± 0.1	2.4 ± 0.1
	Glc	1.9 ± 0.3	1.5 ± 0.1
	Xyl	2.3 ± 0.1	2.1 ± 0.1
Disaccharide	Rha-GlcA	-	-
	Total	36.4 ± 2.4	39.0 ± 1.9

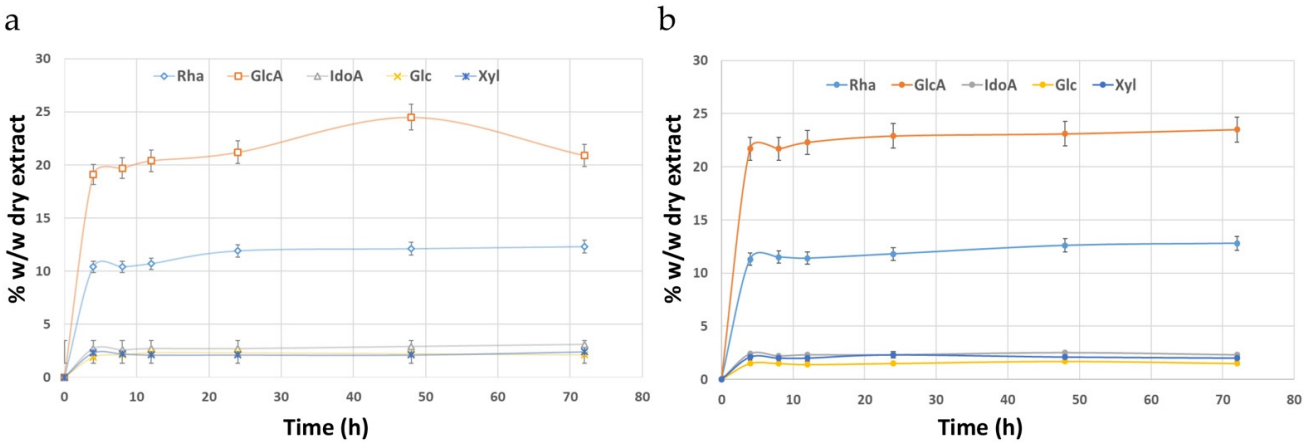

Figure 2. Methanolysis kinetics expressed as number of monosaccharides released over time using (**a**) 2 M HCl and (**b**) 3 M HCl. The data are reported as the average ± SD (n = 3).

With 2 M and 3 M HCl, the total release of monosaccharides after 4 h of reaction was the lowest recorded. As reported by Costa et al. [38], the trend could be related to the degradation of some residues, in particular GlcA, which occurs at the initial stage

of the reaction due to its low stability in acidic environments. This statement is also supported by published studies claiming that HCl is mainly consumed in the first 12 h of the methanolysis [39,40]. As reported by [41], in the first 4 h of the reaction, more acid-sensitive monosaccharides are released, and may further undergo degradation, while starting from 6 h into methanolysis, the break-up of more resistive and protected linkages takes place with an increment in the number of monosaccharides released. It can be seen that the increase in the content of the two main monosaccharides (Rha and GlcA) is linked to the reduction in the Rha-GlcA disaccharide due to the cleavage of the aldo-glucuronosyl linkage in the ulvanobiuronic acid [42].

A higher number of released monosaccharides was observed for a reaction time greater than 24 h in 3 M HCl. Specifically, a noticeable increase could be observed in GlcA and Rha residues while a minor one could be seen in IdoA, Glc and Xyl units. The optimum condition in which the highest release of monosaccharides takes place was established at 48 h in both extracts.

Methanolysis in 2 M HCl/MeOH for 4 h at 100 °C, performed for each polysaccharide extract from *U. ohnoi*, represents an optimal compromise for the effective cleavage of chemical links between the monosaccharide units and prevents degradation after release, as reported by Kidgell et al. [43].

3.2. Microbiological Tests

Obtained raw extracts were finally tested for their biological activities.

3.2.1. Antibacterial Activity

None of the algal extracts showed antibacterial activity at the concentration of 400 μg mL^{-1}, confirming the results previously reported [14,26].

3.2.2. Antibiofilm Activity

The effects of algal extracts at concentrations of 400 μg mL^{-1} on the biofilm formation are reported in Figure 3.

Neither extract (CP and IP) from *U. ohnoi* possessed any relevant antibiofilm activity, either against *Pseudomonas aeruginosa* ATCC 27853 or *Staphylococcus aureus* ATCC 29213 (Figure 3).

The *A. subulata* IP extract reduced the *P. aeruginosa* biofilm by 56%, whereas the *A. subulata* CP extract reduced the *P. aeruginosa* biofilm by 50% (Figure 3a). Neither *A. subulata* extracts, processed with different methodologies, showed a significant difference. The *Sargassum muticum* CP extract strongly reduced the *P. aeruginosa* biofilm (53%) while its IP counterpart exerted a lower effect (38%).

The *Staphylococcus aureus* biofilm was mainly reduced (45%) by the *A. subulata* CP extract (Figure 3b), while the *A. subulata* IP extract exhibited a lower antibiofilm activity (36%) against *S. aureus*. On the contrary, the *S. muticum* IP extract possessed a greater antibiofilm activity (34%) than that obtained with CP extraction (18%).

3.2.3. Emulsification Activity

The emulsifying activity of the crude extracts from *U. ohnoi*, *A. subulata* and *S. muticum* is reported in Table 9.

All the algal extracts possessed emulsifying activity, while at different levels.

Although emulsification indices (E_{24}) of both *U. ohnoi* extracts were lesser than those of extracts of other algae, they exhibited a significant difference between CP and IP methods.

Emulsifying activity of polysaccharides extracted from *A. subulata* and *S. muticum* was higher than that of Triton X, with higher values observed for IP extracts than CP extracts, and the *S. muticum* IP extract had the highest.

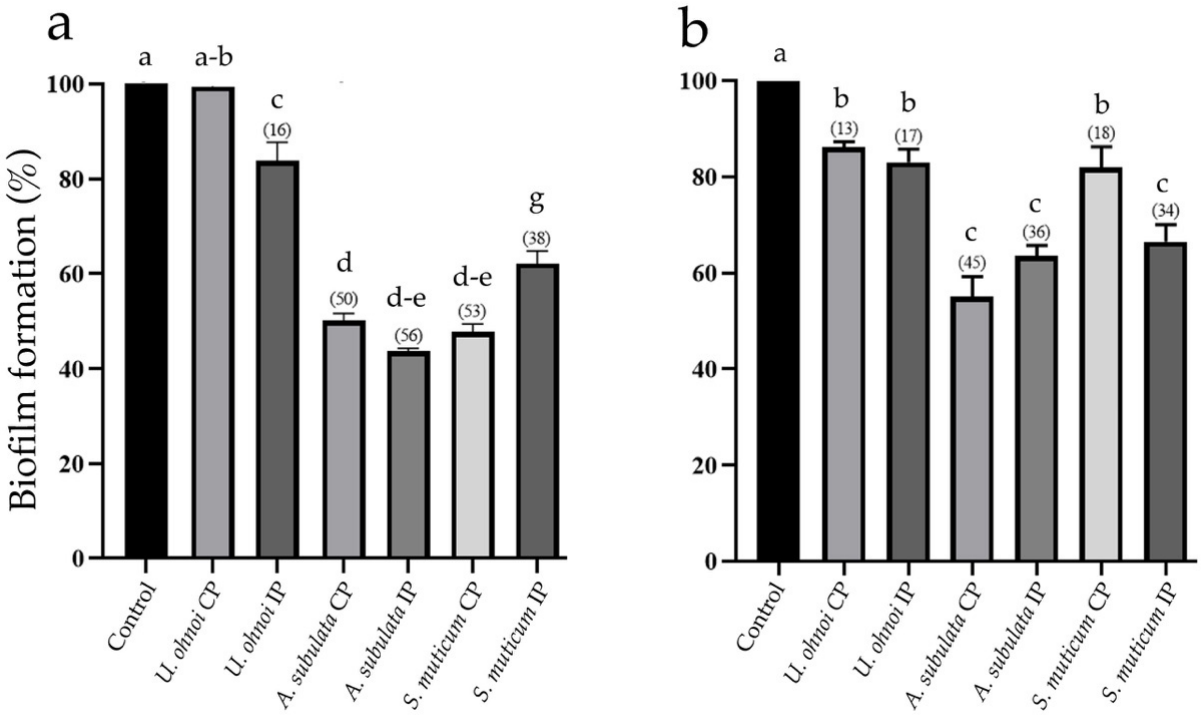

Figure 3. Biofilm formation (%) of (**a**) *Pseudomonas aeruginosa* ATCC 27853 and (**b**) *Staphylococcus aureus* ATCC 29213 in the absence (control, C) or in the presence of the crude extract from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum* (400 µg mL^{-1} *w*/*v*) obtained using the traditional (CP) and innovative (IP) extraction methods. Data represent mean ± SD for six replicates (*n* = 6). The lowercase letters above the bars denote groups that were found to be significantly different after ANOVA followed by Tukey test. In brackets are data on biofilm reduction as a percentage.

Table 9. Emulsification index (E_{24}) of crude extracts, both from conventional (CP) and innovative (IP) methods, from *Ulva ohnoi*, *Agardhiella subulata* and *Sargassum muticum*. Triton X was used as a positive control at the concentration of 0.05% *w*/*v*. The data are expressed as the average ± SD (*n* = 3). The letters denote groups that were found to be significantly different after ANOVA followed by Tukey test.

Extract	E_{24}
Ulva ohnoi CP	22.5 ± 0.6 ^{a}
Ulva ohnoi IP	38.1 ± 0.5 ^{b}
Agardhiella subulata CP	72.5 ± 0.2 ^{c}
Agardhiella subulata IP	75.0 ± 0.3 ^{c}
Sargassum muticum CP	73.0 ± 0.4 ^{c}
Sargassum muticum IP	89.5 ± 0.9 ^{d}
TritonX	70.5 ± 0.6 ^{c}

3.2.4. Coating Assay

The ability of the extracts to inhibit the early adhesion to polystyrene surfaces of *P. aeruginosa* and *S. aureus* after 18 h is reported in Table 10.

All extracts showed at least a moderate ability to inhibit the adhesion of *P. aeruginosa* and *S. aureus* to polystyrene. An exception was represented by *U. ohnoi*, whose CP extract showed no activity against adhesion of both microorganisms, while the IP extract had a moderate activity only against *P. aeruginosa* adhesion.

Full inhibition of bacterial cell adhesion was observed only for *A. subulata* and *S. muticum* CP extracts against *P. aeruginosa*. More investigations are necessary to elucidate the rationale behind such variations and whether they could be related to polymer fragmentation due to microwaves.

Table 10. Ability to inhibit the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to poly-styrene surfaces precoated with polysaccharides from *Ulva ohnoi*, *Agardhiella subulata* and *Sargasssum muticum* (400 μg mL^{-1} *w*/*v*) extracted using both traditional (CP) and innovative (IP) methods. Uncoated polystyrene surfaces were used as control (C).

Extract	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Ulva ohnoi CP	–	–
Ulva ohnoi IP	+	–
Agardhiella subulata CP	+++	+
Agardhiella subulata IP	+	+
Sargassum muticum CP	+++	+
Sargassum muticum IP	+	+
Control	–	–

(–) = negative inhibition of adhesion to polystyrene. (+) = moderate inhibition of adhesion to polystyrene. (+++) = inhibition of adhesion to polystyrene.

4. Conclusions

The main goal of the present work was to elucidate the influence of the extraction methodology on the chemical composition and structure of polysaccharides from different macroalgae. Possible alterations in the chemical structure in terms of molecular weight and the monosaccharide composition, specifically for the lesser-known ulvans, were investigated. All of these characteristics define the chemical, physical and, therefore, biological properties of biopolymers.

Polysaccharide extraction from various sources is the most important task in the investigation and application of bioactive polysaccharides, and the chosen protocol may significantly influence the final product. The present experimental plan was meant to compare conventional (CP) and innovative (IP) microwave-assisted polysaccharide extraction protocols, focusing on extraction times, polysaccharide yield, chemical composition and, finally, biological activities of raw extracts. The main objective was to design a sustainable process in terms of energy and time savings.

Under the tested conditions, extraction efficacy was likely dependent on the physicochemical polysaccharide properties. Nevertheless, even if *Agardhiella subulata* and *Sargassum muticum* gave comparable yields of isolated polymers in both tested methods, it is noteworthy that the obtained polysaccharides almost tripled in IP extraction from *Ulva ohnoi*.

As proved by spectroscopy analyses (FTIR-ATR), the use of microwaves, under the tested settings, did not affect the chemical structure of the polysaccharides extracted, even if hypochromic effects were observed in all IP extracts. However, gel permeation chromatography (GPC) allowed us to hypothesize that microwaves in the IP could produce a reduction in the molecular weight of extracts from *A. subulata* and *S. muticum* (bimodal distribution), but not from *U. ohnoi* (monomodal distribution). This hypothesis confirms the literature data, which some authors use to posit that microwave treatment could result in a dishomogeneous distribution of temperature with local increases in the extraction mix [44,45].

To evaluate any differences in biological properties of crude extracts obtained from the two protocols, we tested their antibacterial and antibiofilm activities against *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213, our model microorganisms. Without any antibacterial activity, all the extracts possessed antibiofilm activity against *P. aeruginosa* and *S. aureus*, at different levels. The antiadhesive and emulsi-

fying properties of raw extracts could inhibit the bacterial biofilm formation, likely thanks to their ability to modify the surface properties. Comparing the CP and the IP, the highest differences were observed in the emulsification activity, with the IP extracts from *S. muticum* and *U. ohnoi* being more active than their CP counterparts, suggesting that modifications in their structural composition might occur in extraction processing.

Obtained results indicate that the innovative method of extraction could be used as an alternative to the conventional one to achieve bacterial antiadhesives and emulsifiers in different applications. Nevertheless, considering the large number of variables implicated, such as the algal taxon and the polysaccharide type, more investigations are needed to clarify the entire extraction process.

Finally, we chose to carry out the entire experimentation using natural populations of invasive algae rather than cultivated species in order to propose the valorization of unwanted biomasses, which are commonly treated as a waste, converting them into a prized resource and setting the framework for a sustainable process.

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3.2 Hydrochar Obtained by Hydrothermal Carbonization of macroalgae *Sargassum muticum*

Manuscript in preparation

This research aimed to define a green protocol to obtain hydrochar by Hydrothermal Carbonization (HTC) from the brown algae *Sargassum muticum*. Process conditions like temperature (180–300 °C) and reaction time (60–300 min) were investigated in order to study the differences among the obtained carbon materials and to find the optimum conditions that maximize yield. The highest yield of hydrochar was obtained at 180 °C of reaction temperature and 180 min of residence time. The obtained hydrochar was characterized and showed interesting properties that would guarantee a variety application.

3.3 Improvement of the growth of *Phaseolus vulgaris* through aqueous byproduct of hydrothermal carbonization of brown algal biomass

Manuscript in preparation

The HTC process produces a discarded liquid phase termed aqueous HTC liquid (AHL) that may be reused in agricultural. The present study is aimed at investigating the effects of seed primining treatments with three different AHL solutions obtained from three different HTC experimental procedures on seed germination and plant growth and productivity of Phaseolus vulgaris 'Borlotto'. To disentangle the osmotic effects from the use of AHL, isotonic solutions of polyethilenglycole (PEG) 6000 were also tested. Seed germination has been unaffected by AHL-seed priming treatment. Conversely, PEG-treated samples showed significant lower seed germination success. AHL-treated samples showed changes in whole plant biomass. In fact, a higher shoot biomass was recorded especially in AHL180 samples. By contrast, AHL240 and AHL300 samples showed higher root biomass. These results were the consequence of changes in stomatal density and then photosynthesis rate and water use efficiency. Overall, present findings strongly support the hypothesis of AHL solutions reuse in agriculture, especially if obtained by the seaweed feedstock, in the framework of resource management and circular green economy.

4 Uses of extracts and by-product

4.1 *In vitro* evaluation of antibiofilm activity of crude extracts from macroalgae against pathogens relevant in aquaculture

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In vitro evaluation of antibiofilm activity of crude extracts from macroalgae against pathogens relevant in aquaculture

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ABSTRACT

The exploitation of macroalgal biomass could provide novel environment-friendly molecules to prevent bacterial biofilm formation. Crude extracts, or polysaccharides, from the DNA-barcoded macroalgae *Chaetomorpha aerea* (Chlorophyta), and *Agardhiella subulata* and *Hypnea cornuta* (Rhodophyta) collected from the coastal brackish Lake of Ganzirri (Italy) were evaluated for their effects on biofilm formation of *Listonella anguillarum, Vibrio harveyi, V. parahaemolyticus* and *Photobacterium damselae* subsp. *piscicida*.

All bacterial strains were able to produce biofilm on polystyrene surfaces at different extents. Without exerting antibacterial activity, the crude polysaccharides showed dose-dependent inhibitory activity on the biofilm formation. At the highest concentration (400 μg/ml), the strongest reduction of biofilm formation was observed in the presence of *C. aerea* and *A. subulata* extracts against *V. harveyi* (59 and 53%, respectively), followed by the *H. cornuta* extract against *P. piscicida* (52%) and *V. parahaemolyticus* (28%). The extract from *A. subulata* was also active against the biofilm formation of *P. piscicida* (48%) and *L. anguillarum* (33%).

As resulted by the surface coating assay and the microbial adhesion onto hydrocarbons, the algal extracts were able to inhibit the adhesion of pathogens and modify their cell-surface properties and hydrophobicity. Moreover, all the extracts possessed high emulsifying activity (E_{24} up to 70%) and stabilizing capacity.

Based on two different *in vivo* assays, using *Artemia salina* and *Danio rerio,* all the extracts showed no potential toxicity for aquaculture application.

Our results suggest that the antibiofilm activity of algal extracts is related to the inhibition of the early bacterial adhesion. Consequently, these non-toxic and biodegradable polysaccharides, with surfactant and anti-adhesive properties, could be proposed to prevent biofilms in aquaculture.

1. Introduction

Bacterial biofilms are dense cellular aggregates of surface-attached microorganisms encased in a self-synthesized extracellular polymeric matrix (Costerton et al., 1999). In almost any submerged surfaces, both natural and man-made systems, more than 90% of microorganisms form biofilms, which are usually produced by bacteria to escape from unfavorable conditions, such as the presence of predators, microbial inhibitors (*i.e.*, antibiotics, disinfectants and host defenses), the lack of nutrients and further stressors (Karunasagar et al., 1994). The biofilm formation starts with an initial attachment of free-living bacteria and the adhesion to abiotic and biotic surfaces becomes irreversible when the cells secrete exopolymers with the subsequent development and stabilization of biofilm architecture (Monds and O'Toole, 2009). The early bacterial attachment to the surfaces is the most crucial step in the biofilm formation, since environmental physicochemical conditions (temperature, pH, salinity, *etc.*), surface properties (substrate type, surface roughness and chemical composition), cell-surfaces charges and hydrophobicity may compromise the entire biofilm establishment (Balebona et al., 2001; Tuson and Weibel, 2013).

The highly tolerance and persistent nature of biofilms cause severe loss of production with high economic impact in a wide variety of

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Table 1
List of the algal samples used in this study and BOLD accession numbers.

Phylum	Species	Code	BOLD accession number
Chlorophyta	*Chaetomorpha aerea*	POL004	GRAPP001–17
Rhodophyta	*Agardhiella subulata*	POL046	ITRED008–10
Rhodophyta	*Hypnea cornuta*	POL052	ITRED081–13

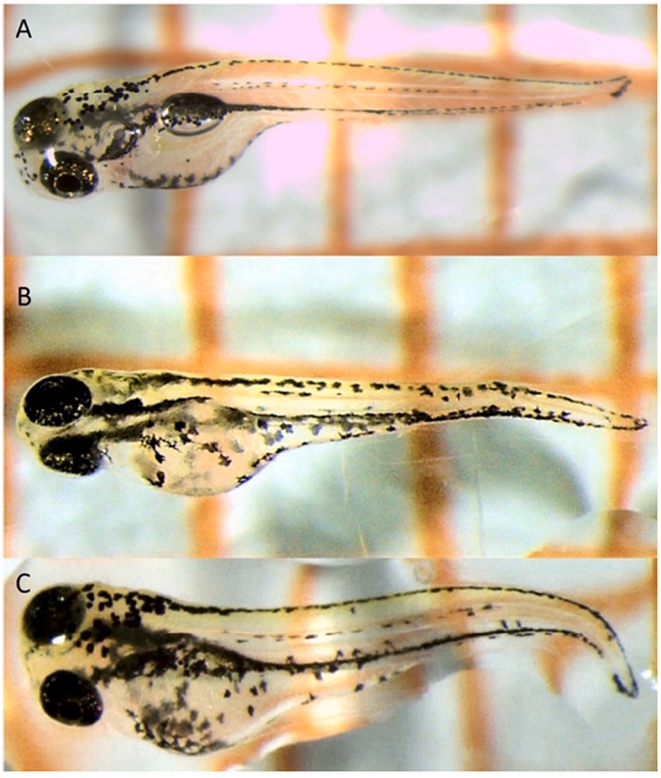

Fig. 1. Zebrafish larvae at 96 h; (A) Control group; *Hypnea cornuta* extract induced tail deformities (400 μg/ml): (B) Zebrafish larvae with scoliosis of tail (C) Zebrafish larvae with hooked tail.

Table 2
Biofilm formation of *Vibrio harveyi* G5, *Vibrio parahaemolyticus* L12G, *Listonella anguillarum* Van and *Photobacterium piscicida* Pdp expressed as OD_{585}nm. Each value was obtained by six replicates. Standard deviations are reported ([a]: incubation for 48 h; [b]: incubation for 18 h).

Strain	Biofilm (OD_{585nm})
V. harveyi G5	2.8 ± 0.1^{a}
V. parahaemolyticus L12G	1.6 ± 0.1^{a}
L. anguillarum Van	3.2 ± 0.0^{b}
P. piscicida Pdp	2.4 ± 0.1^{b}

sectors, including medicine, food or feed industry, agriculture and aquaculture (Bourne et al., 2006). Bacterial diseases represent one of the largest constrains in aquaculture, since bacterial pathogens living in biofilm state are highly tolerant to disinfectants and mechanical removal, and are 10–1000-fold more resistant to antibiotic treatment than free-living cells (Hughes and Webber, 2017). Therefore, biofilms are notoriously difficult to eradicate and could also represent a reservoir for pathogens, causing chronic infections and recurring diseases (King et al., 2004; Balcàzar et al., 2015; Cai and Arias, 2017). Consequently, the exploration of novel anti-biofilm strategies, aimed at searching for novel compounds to prevent biofilm, currently constitutes an important field of investigation.

Diseases in fish and shellfish are mainly caused by bacteria belonging to the *Vibrionaceae* family, which represent the major threats to aquaculture sector, particularly occurring in the warm waters of Asia, southern Europe, and South America. Several halophilic vibrios, such as *Listonella anguillarum* (formerly *Vibrio anguillarum*), *Vibrio harveyi* and *Vibrio parahaemolyticus*, which are ubiquitous in freshwaters and marine environments, are responsible for the most common vibriosis in fish and shellfish, and they are cause of enteric pathologies in humans, primary and secondary septicemia, and infections in skin wounds exposed to seawaters or associated with fish handling (Thompson et al., 2005; Toranzo et al., 2005; Austin and Zhang, 2006; Nishibuchi and De Paola, 2012; Akram et al., 2015). *Vibrio* spp. have been associated in summer mortality of bivalve mollusks (oysters, clams and scallops). *L. anguillarum* and *V. harveyi* are responsible for larval vibriosis in different mollusk species (Paillard et al., 2004; Garnier et al., 2007). *L. anguillarum* has been also recognized among the main significant pathogens for a variety of fish, mainly of seabass (*Dicentrarchus labrax*), crustaceans and bivalves (Hickey and Lee, 2017). *V. harveyi* is considered a primary pathogen of a wide range of cultured species, including marine fish, causing several diseases such as vasculitis, gastroenteritis and eye-lesions, as well as penaeid shrimps and mollusks (such as *Haliotis tuberculata)*, being cause of mass mortality in aquaculture farms (Austin and Zhang, 2006; Zhang et al., 2014, 2020). *V. harveyi* has been identified as an emerging major concern for seabass (*D. labrax*) aquaculture, since there are no specific and effective prophylactic control measures until now (Vendramin et al., 2016; Dadar et al., 2017). This species has been retrieved in free-floating state, in biofilms attached to biotic and abiotic surfaces, or in symbiotic and host-pathogen interactions (Montánchez and Kaberdin, 2020). *Photobacterium damselae* subsp. *piscicida* (previously *Pasteurella piscicida*) is known as the causative agent of a severe disease called photobacteriosis in cultured fish, including seabass and *Senegalese sole* (Romalde, 2002; Zorrilla et al., 2003), crustaceans, mollusks and large sea mammals. *P. piscicida* is considered resistant to most of the antibiotics used in the treatment of fish pseudo-tuberculosis (Romalde, 2002).

Seaweeds are important sources of various compounds that possess a wide spectrum of activities, such as antioxidant, antimicrobial, anti-inflammatory, and anticancer (Genovese et al., 2012; Marino et al., 2016; Rizzo et al., 2017; Bilal and Iqbal, 2020 and therein references). Marine algae contain large amounts (accounting from 4 to 76% of dry weight) of polysaccharides (including alginates, carrageenans, agar, laminarans, fucoidans and ulvans) with important structural and storage functions (Murata and Nakazoe, 2001; Kumar et al., 2008; Holdt and Kraan, 2011). Polysaccharides are essential to basic cellular processes, such as cell growth, thallus development and water potential balancing in response to environmental stresses (Hurd et al., 2014). Structural polysaccharides add consistency to the algal cell walls, providing also a certain amount of elasticity necessary in the aquatic environment. They provide protection for the cells against desiccation by binding water and they may serve as a kind of ion-exchange material. Variability in the qualitative and quantitative production of polysaccharides, also within the same algal genus, may depend on different ecological factors, such as geographical distribution, and physiological conditions (Genovese et al., 2012; Armeli Minicante et al., 2016; Pérez et al., 2016).

Macroalgae inhabiting transitional environments, such as that of the coastal Lake of Ganzirri (Messina, Italy), produce large amounts of biomass, which needs to be periodically removed and treated as a waste (Genovese et al., 2012; Rizzo et al., 2017). The collection of invasive algal species from the environment may represent a double chance both for the mitigation of their negative impacts, and transforming waste into valuable resource of new bioactive compounds with biotechnological relevance in aquaculture.

In an ongoing screening for novel environment-friendly antibiofilm molecules, in the present study water extracts, or polysaccharides, from *Chaetomorpha aerea* (Dillwyn) Kützing (Chlorophyta), *Agardhiella subulata* (C. Agardh) Kraft & MJ Wynne (Rhodophyta), and *Hypnea cornuta* (Kützing) J. Agardh (Rhodophyta), collected from the brackish Lake of

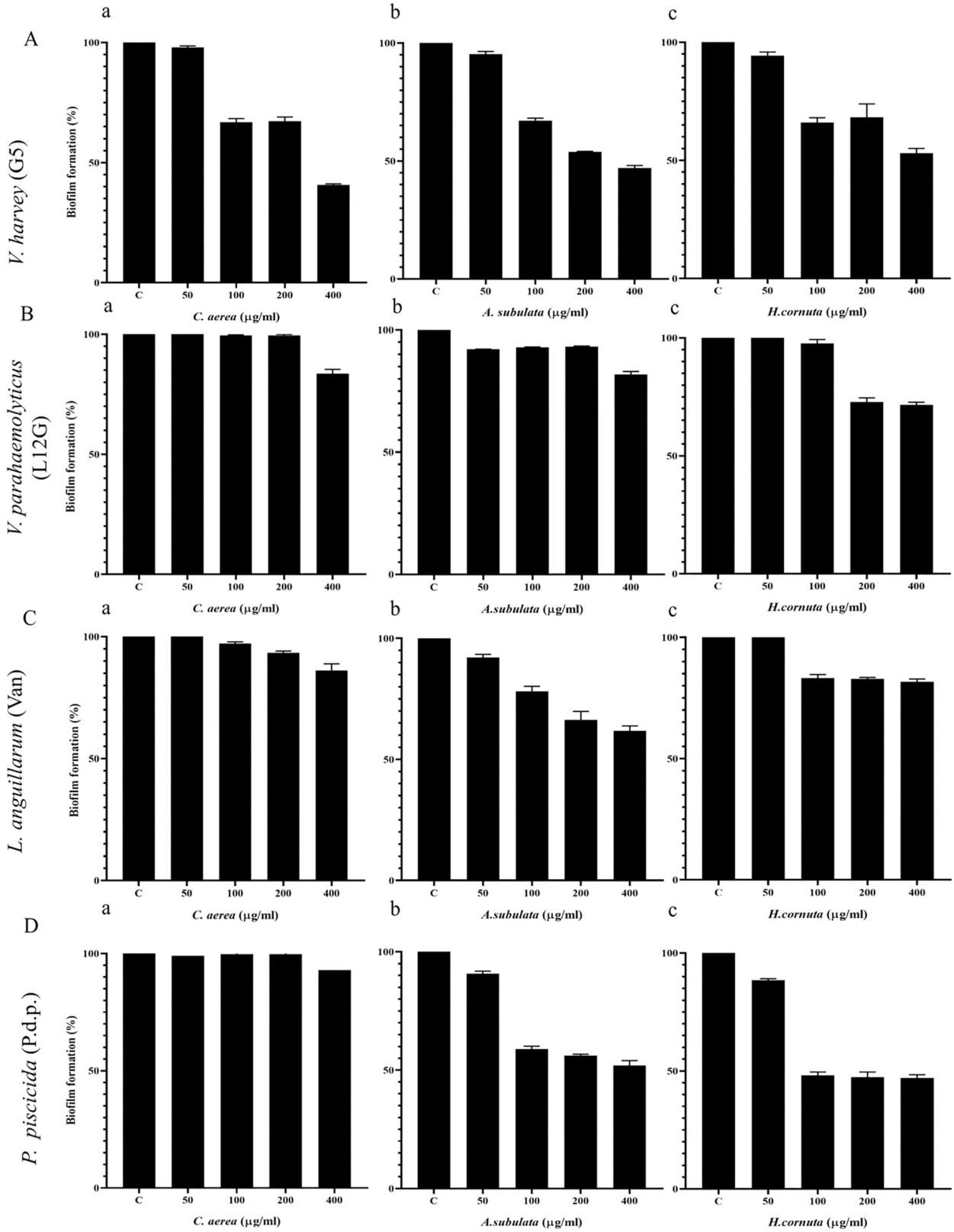

Fig. 2. Biofilm formation (%) by A) *Vibrio harveyi* G5, B) *Vibrio parahaemolyticus* L12G, C) *Listonella anguillarum* Van and D) *Photobacterium piscicida* Pdp in absence (control, C) or in the presence of the crude extract from a) *Chaetomorpha aerea,* b) *Agardhiella subulata* and c) *Hypnea cornuta* at increasing concentrations (from 50 to 400 μg/ml). Data represent mean ± SD for seven replicates ($n = 6$).

Table 3
Bacterial growth in the presence of algal extracts at different concentrations (from 50 to 400 μl/ml) spectrophotometrically evaluated (OD_{600mm}).

Extract from	Strain	Control	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml
Chaetomorpha aerea	*V. harveyi G5*	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.0
	V. parahaemolyticus L12G	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0
	L.anguillarum Van	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1
	P. piscicida Pdp	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.1
Agardhiella subulata	*V. harveyi* G5	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.0
	V. parahaemolyticus L12G	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
	L. anguillarum Van	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0
	P. piscicida Pdp	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
Hypnea cornuta	*V. harveyi* G5	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.0
	V. parahaemolyticus L12G	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
	L. anguillarum Van	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.1
	P. piscicida Pdp	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0

Table 4
Vibrio harveyi G5, *Vibrio parahaemolyticus* L12G, *Listonella anguillarum* Van, *Photobacterium piscicida* Pdp adhesion to polystyrene in absence (Control) or in pre-coated surfaces with crude extracts (400 μg/ml) from *Chaetomorpha aerea, Agardhiella subulata* and *Hypnea cornuta*.

Crude extracts	*V. harveyi* G5	*V.parahaemolyticus* L12G	*L. anguillarum* Van	*P. piscicida* Pdp
Control	−	−	−	−
C. aerea	+	−	−	−
A. subulata	+	−	+	+
H. cornuta	+	−/+	−	+

(−) = negative capacity of inhibition of adhesion to polystyrene
(−/+) = moderate inhibition of adhesion to polystyrene
(+) = inhibition of adhesion to polystyrene

Ganzirri (Messina, Italy), were tested for their activity against the biofilm formation of four pathogens relevant for aquaculture (*L. anguillarum, V. harveyi, V. parahaemolyticus* and *P. damselae* subsp. *piscicida*). Molecular methods (*i.e.*, DNA barcoding sequencing) was used to univocally identify the algal species with promising applications. The eco-toxicity of the algal extracts for *Artemia salina* and *Danio rerio* was also investigated.

2. Materials and methods

2.1. Sample collection

Samples of *C. aerea* (Dillwyn) Kützing (Chlorophyta) and of two Rhodophyta, *A. subulata* (C. Agardh) Kraft & MJ Wynne and *H. cornuta* (Kützing) J. Agardh, were collected from the brackish Lake of Ganzirri in the Cape Peloro lagoon of Messina (Italy). All species used in the present investigation have isomorphic life cycles, with alternating gametophytic and sporophytic phases, which thrive in mixed populations and are distinguishable only through fine reproductive aspects by trained experts. In the perspective of potential economic exploitation, we decided to use natural populations and to process mixed haploid and diploid batches.

Fresh samples were washed with sterile seawater and manually cleaned of epiphytes. From each sample, a part was dried in silica gel and stored at −20 °C for molecular identification and the rest was oven-dried at 40 °C for 48 h for the polysaccharidic extraction.

2.2. Algal extracts

Dried samples (100 g) of macroalgae were processed to obtain crude polysaccharides, as detailed in Armeli Minicante et al. (2016) with some modifications, and slightly different protocols were performed in relation to the class of polysaccharides to be obtained (*i.e.,* carrageens and sulphated polysaccharides). For the red algae, dried samples were soaked in NaOH 80 g/l (about 1:1 w:v) at 70 °C in oven for 2 h, then filtered through cotton-net and washed in distilled water. Subsequently, polysaccharides were extracted from thalli by soaking in distilled water (about 1:10 w:v) at 70 °C for 24 h. Finally, exhausted thalli were separated and polysaccharides were precipitated adding an equal volume of absolute ethanol to the mixture and then centrifuged at 4000 rpm. Obtained crude extracts were dried at 40 °C overnight. The extraction from *C. aerea* was performed in HCl solution (pH 2) at 70 °C for 3 h, without the pretreatment in NaOH.

2.3. DNA barcoding

DNA barcoding identification was performed according to the protocols described in Miladi et al. (2018) and Manghisi et al. (2019). Selected barcodes were COI-5P for Rhodophyta and LSU D2/D3 for *C. aerea* (Saunders and McDevit, 2012). Sequencing reactions were performed by an external company (Macrogen Europe, The Netherlands). Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd), deposited in BOLD Systems (http://www.boldsystems.org/) and species attributions were performed by the identification engine in BOLD Systems.

2.4. Toxicity assays

2.4.1. Brine shrimp lethality assay

In order to investigate the extracts toxicity, the brine shrimp (*Artemia salina*) bioassay was carried out according to Caputo et al. (2020). Brine shrimp's eggs were purchased by a local pet shop, placed in a hatcher chamber containing artificial seawater, and incubated for 48 h at room temperature with continuous aeration and illumination. Stock solutions (500–4000 μg/ml) of *C. aerea*, *A. subulata*, *H. cornuta* extracts, as well as $K_2Cr_2O_7$ (500 μg/ml) used as a positive control, were prepared in water. After that, 200 μl of each sample/control was seeded in a 24-well plate diluting 1:10 *v*/v in artificial seawater. Ten nauplii per well were added and incubated for 48 h in the same conditions reported above. Surviving larvae without abnormal swimming behavior after 24 h and 48 h were counted by a stereomicroscope (SMZ-171 Series, Motic). One negative control (ten larvae treated with only artificial seawater) was evaluated. Three independent experiments ($n = 10$) were carried out for each treatment. Lethality was calculated using the following equation:

$$\%\ \text{Lethality} = 100 - \frac{\text{slt x } 100}{\text{slcs}}$$

where *slt* were the survival larvae treated with the extracts or $K_2Cr_2O_7$, and *slcs* were the survival larvae treated with artificial

Table 5
Affinity (expressed as percentage) to polar (ethyl acetate and chloroform) and non-polar solvents (decane and exadecane) of *Vibrio harveyi* G5, *Vibrio parahaemolyticus* L12G, *Listonella anguillarum* Van, *Photobacterium piscicida* Pdp cells in not-treated or treated with the crude extracts (400 μg/ml) from *Chaetomorpha aerea, Agardhiella subulata* and *Hypnea cornuta*.

		Polar solvent		Non-polar solvent	
		Ethyl acetate	Chloroform	Decane	Hexadecane
V. harveyi G5	Not treated	48.4 ± 0.6	81.7 ± 0.6	22.0 ± 1.4	11.3 ± 0.5
	C. aerea	82.9 ± 0.3**	44.7 ± 0.3**	13.2 ± 0.6**	11.5 ± 0.6
	A. subulata	26.2 ± 2.8**	79.4 ± 1.4**	3.9 ± 0.2**	9.2 ± 1.4
	H. cornuta	53.9 ± 0.5	83.3 ± 0.7	18.1 ± 0.4	37.3 ± 0.9**
V. parahaemolyticus L12G	Not treated	18.1 ± 2.9	77.9 ± 0.4	19.3 ± 2.8	3.2 ± 0.2
	C. aerea	12.4 ± 1.4	77.4 ± 0.4	26.7 ± 2.8*	23.7 ± 1.2**
	A. subulata	19.9 ± 0.5	61.2 ± 0.3**	14.4 ± 0.6*	3.0 ± 0.9
	H. cornuta	23.2 ± 3.5	85.1 ± 0.3	5.4 ± 0.3**	4.5 ± 0.4
L. anguillarum Van	Not treated	50.4 ± 1.7	56.1 ± 1.8	73.5 ± 2.1	8.7 ± 0.1
	C. aerea	60.6 ± 0.5	58.1 ± 0.4	56.8 ± 0.8**	0.5 ± 0.1**
	A. subulata	28.1 ± 0.1**	7.4 ± 0.6**	27.0 ± 1.7**	1.0 ± 0.4**
	H. cornuta	94.3 ± 2.7**	42.2 ± 0.4**	96.1 ± 1.8**	12.7 ± 1.1
P. piscicida Pdp	Not treated	27.5 ± 0.2	34.5 ± 0.4	27.9 ± 1.1	25.6 ± 0.1
	C. aerea	0.8 ± 0.1**	25.9 ± 0.2*	39.5 ± 0.6*	30.2 ± 1.2
	A. subulata	40.6 ± 0.5**	28.4 ± 0.1	1.2 ± 0.1**	3.7 ± 0.1**
	H. cornuta	26.9 ± 0.1	14.6 ± 0.4**	30.5 ± 0.6	5.1 ± 0.5**

* Significantly different ($p < 0.05$) compared with not treated controls.
** Significantly different ($p < 0.01$) compared with not treated controlls.

Table 6
Emulsifying activity of crude extracts from *Chaetomorpha aerea, Agardhiella subulata* and *Hypnea cornuta* in comparison with Triton X-100.

Extract	E_{24} (%)
C. aerea	95.6 ± 0.6
A. subulata	72.5 ± 0.2
H. cornuta	82.2 ± 0.4
Triton X-100	70.5 ± 0.3

seawater (negative control).

2.4.2. Zebrafish embryo toxicity test

Wild-type (WT) zebrafish (*Danio rerio*) were raised in the Centre for Experimental Fish Pathology (Centro di Ittiopatologia Sperimentale della Sicilia – CISS) at the Department of Veterinary Sciences, University of Messina, Italy. Adult zebrafish were kept in a stand-alone facility (ZebTec, Tecniplast) in a water-controlled conditions: 14/10 h dark/light regimen, Temperature 28 °C, pH 7.5, Conductivity 600 μS/cm. Fish were fed twice a day with Gemma micro 300 (Skretting, Varese, Italia) and *A. salina* at 3% of body weight.

Algal extracts from *A. subulata*, *C. aerea* and *H. cornuta* were dissolved in sterilized embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$; pH 7.3) at the final concentrations of 200 μg/ml and 400 μg/ml for each extract. Control group was kept in only embryo medium and was preserved from any handling over the zebrafish embryo toxicity test (ZFET).

Adult zebrafish were set up in spawning groups at a ratio of two females and one male, and the eggs were collected to carry out the ZFET according to OECD guideline (OECD TG 236, 2013). To determine acute toxicity of the three algal extracts on embryonic stages of zebrafish (*D. rerio*), the Fish Embryo Acute Toxicity (FET) was carried out as described by Busquet et al. (2014). Immediately after fertilization, embryos were bleached as reported by Westerfield (2007), and divided into 24-well plates (LABSOLUTE, Th.Geyer GmbH & Co.KG) (1 embryo per well). The evaluation of the toxicity was estimated by several endpoint: coagulation of the embryo, non-detachment of the tail, lack of somites formation, non-detection of the heartbeat, and the number of hatched embryos were recorded. At the end of the exposure period, acute toxicity was determined, based on a positive outcome in any of the four apical observations recorded, and the lethal concentration (LC_{50}) was calculated.

All the examinations were made every 24 h under stereoscope (Leica M205 C). Embryos were incubated at 26 °C with 10/14 h dark/light regimen, for 96-h post fertilization (hpf). The test solutions/controls were renewed on a daily basis after recording any toxic or lethal effects on the embryos; at least 90% of the volume of each well was removed without touching the embryo and immediately replaced with freshly prepared test solutions at the same concentration (OECD, 2013).

2.5. Bacterial strains

The following pathogens strains were tested: *V. harveyi* (G5), *V. parahaemolyticus* (L12G), *L. anguillarum* (Van) and *P. damselae* subsp. *piscicida* (Pdp). These strains were previously isolated from local marine and brackish environments and identified using standard biochemical and physiological characterization, and phylogenetic analysis, as previously reported (Gugliandolo et al., 2011; Genovese et al., 2012). Lab strains are maintained at −20 °C in Tryptone Soy Broth (TSB, Oxoid) supplemented with 1% of NaCl (Oxoid) (TSB1) and 50% (v/v) glycerol. All strains are housed in the Culture Collection of Microbial Ecology laboratory at the Department of Chemistry, Biological, Pharmaceutical and Environmental Sciences, University of Messina (Italy).

2.6. Biofilm formation of bacterial strains and antibiofilm activity of algal extracts

2.6.1. Biofilm formation

The ability to produce biofilm of the four strains (*V. harveyi* G5, *V. parahaemolyticus* L12G, *L. anguillarum* Van, and *P. damselae* subsp. *piscicida* Pdp) was evaluated in 96-well polystyrene microtiter plates (Falcon no. 353047) (Coffey and Anderson, 2014). Overnight cultures grown in TSB1 were diluted at $OD_{600} = 0.1$ (equivalent to 1.5×10^8 bacteria/ml) with fresh sterile medium and each well of microtiter plates was filled (12 replicates) with aliquots of 200 μl. The plates were incubated at 25 °C for 18 h for *L. anguillarum* Van and *V. parahaemolyticus* L12G (Enos-Berlage et al., 2005) or 48 h for *V. harveyi* G5 (Vinay et al., 2019) and *P. piscicida* Pdp (Tan et al., 2017), without shaking for a stable biofilm formation.

Nonadherent bacteria were removed by washing 3 times with distilled sterile water, by gentle aspiration. Adherent bacteria (biofilms) were stained with 0.1% crystal violet solution (w/v) for 25 min, as

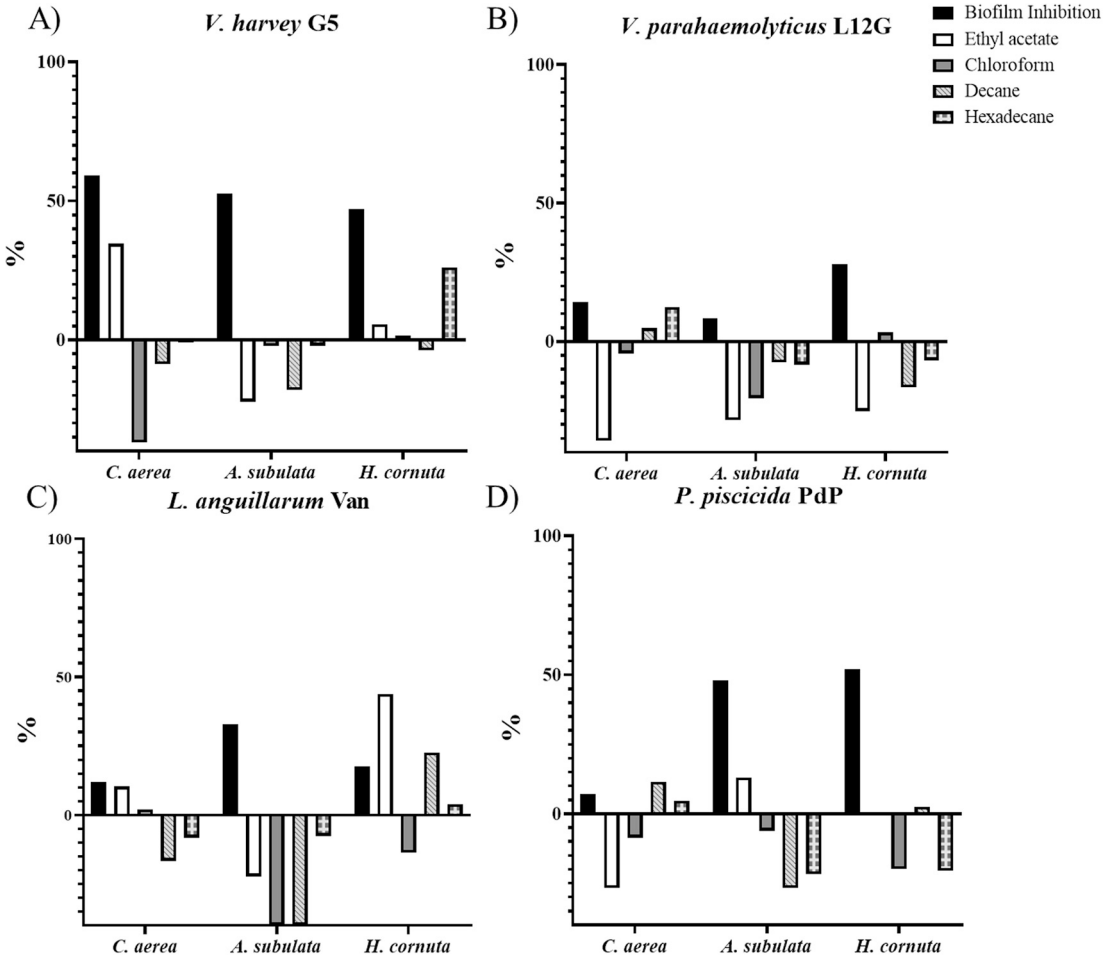

Fig. 3. Biofilm inhibition (black bars) and affinity variations (%Affinity treated - %Affinity not-treated) to hydrocarbons (ethyl acetate, chloroform, decane and hexadecane) in the presence of crude extracts (400 mg/ml) obtained from *Chaetomorpha aerea*, *Agardhiella subulata* and *Hypnea cornuta*.

reported by O'Toole (2011). Excess stain was removed, and the plates were washed (5 times) and airdried (for 15 min). The stained biofilms were solubilized with absolute ethanol. Biofilm mass, estimated by the level of crystal violet present in the de-staining solution, was spectrophotometrically (OD = 585 nm) evaluated, using a microtiter plate reader (Multiskan GO, Thermo Scientific, USA). Each data point from six replicated microwells was averaged, and the standard deviation was calculated (SD).

2.6.2. Antibiofilm activity

To evaluate the ability of each algal extract to inhibit biofilm formation of the pathogen strains tested, an overnight bacterial culture in TSB1 (180 µl) ($OD_{600nm} = 0.1$, equivalent to 1.5×10^8 bacteria/ml) was added to a 96-well polystyrene microtiter plates, in six replicates, together with aliquot (20 µl) of each algal extract solution in PBS, to obtain final concentrations ranging from 50 to 400 µg/ml. The biofilm formation of the pathogenic strains in the presence of algal extracts or PBS (20 µl) used as control, was spectrophotometrically assessed as described above. The reduction of biofilm formation was expressed as inhibition of biofilm (%) by applying the following formula:

$$\frac{OD_{585nm}\ \text{Control} - OD_{585nm}\ \text{Sample}}{OD_{585nm}\ \text{Control}} * 100$$

Each data point was averaged from six replicated wells and the SD was calculated.

2.7. Antibacterial assay of algal extracts

Overnight culture in TSB1 of each pathogen (OD_{600}= 0.1) (180 µl) was distributed in 96-well polystyrene microtiter plates (six replicates), in the presence of each algal extract at 400 µg/ml final concentration in PBS (20 µl) or with PBS (20 µl) used as control. The microplates were incubated at 25 °C for 24 h without shaking and OD_{600} values were registered.

Antibacterial activity was evaluated by using the standard disk diffusion method (Kirby Bauer test), as accepted by the National committee for Clinical laboratory Standard (NCCLS 2000).

Strains were overnight grown onto plates of Tryptone Soya Agar (TSA, Oxoid) amended with 1% (w/v) of NaCl (TSA1), for 24 h at 25 °C. Each strain was suspended in 3 ml of 0.9% NaCl solution (OD_{600nm} = 0.1) and aliquots of each suspension (100 µl) were inoculated onto triplicate plates of TSA1. Each algal extract (20 mg) was dissolved in 1000 µl of sterile distilled water and 20 µl of each solution were applied to sterile filter paper disks (6 mm in diameter, Oxoid). After evaporation, the disks (containing each 400 µg) were placed onto inoculated plates. Plates were incubated overnight at 25 °C. The diameter of complete inhibition zone was measured and means and standard deviations (n = 3) were calculated.

2.8. Surface coating assay

A volume of 10 µl of 400 µg/ml of each extract in PBS saline buffer, or 10 µl of PBS used as control, was transferred to the center of each well in

24-well polystyrene microtiter plates (Falcon no. 353047) (Karwacki et al., 2013). The plates were incubated at 25 °C for 30 min to allow the complete evaporation of the liquid. The wells were filled with 1 ml of each overnight bacterial culture in TSB1 (diluted to contain 10^5 CFU/ml). After 18 h at 25 °C, the cultures were gently removed, wells were washed with tap water and stained with 1 ml 0.1 % crystal violet solution. Stained biofilms were rinsed with tap water and air dried.

2.9. Microbial cell-surface properties and hydrophobicity

Analysis of cell-surface properties, based on bacterial adherence to hydrocarbons, was measured according to Bellon-Fontaine et al. (1996). Bacterial cells grown overnight in TSB1 at 25 °C, were harvested and washed twice with PBS. Aliquots (2 ml) of each bacterial suspension were treated with each algal extract solution (400 μg/ml in PBS) for 30 min, or with PBS used as control. Suspensions were centrifuged at 6000 rpm for 10 min and resuspended in PBS with OD_{400nm} comprised from 0.5 to 0.7 (A0). An aliquot of 3 ml of each bacterial suspension was added to each tube containing 0.4 ml of the following hydrophobic solvents: ethyl acetate (Sigma-Aldrich), a strong basic solvent; chloroform (Sigma-Aldrich), an acidic solvent which exhibits negligible basic character; decane (Sigma-Aldrich), as a non-polar solvent having intermolecular attraction comparable to that of ethyl acetate, and hexadecane (Sigma-Aldrich), as a non-polar solvent having intermolecular attraction comparable to that of chloroform. After vigorous agitation by vortex, phases were allowed to separate for 10 min at 30 °C and the OD_{400nm} of the aqueous phase was measured (A1). The percentage of affinity for hydrocarbons was calculated as follows:

$$\% \text{ Affinity} = \frac{A0 - A1}{A0} \times 100$$

All assays are representative of three independent experiments.

2.10. Emulsifying activity

The emulsifying activity of crude algal extracts was evaluated according to the method described by Mata et al. (2006). Each crude, lyophilized extract was dissolved in 2 ml distilled water (0.05%, w/v), mixed with an equal volume of kerosene in a glass tube (10 cm high and 1 cm in diameter) and stirred at high speed in the vortex for 2 min. The emulsion and aqueous layers were measured after 24 h, and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture, and multiplying by 100 (Satpute et al., 2008). Triton X-100 (Sigma-Aldrich) was used as positive-control surfactant.

3. Results

3.1. Algal extracts and DNA barcoding identification

DNA barcode sequences were obtained for all tested algae and submitted to BOLD System, allowing a permanent genetic labelling of algal species. BOLD-accession numbers are reported in Table 1.

3.2. Toxicity assays

3.2.1. Brine shrimp lethality assay

The *A. salina* assay is a useful and rapid screening tool to evaluate the toxicity of plant complexes or pure compounds on *in vivo* model. *A. salina* is a marine zooplanktonic organism, which represents the gold standard in toxicological assays, due to its easy cultivation, availability, low cost and adaptation to adverse conditions (Danabas et al., 2020).

C. aerea, *A. subulata* and *H. cornuta* extracts were tested at different concentrations (ranging from 50 to 400 μg/ml) after 24 and 48 h treatments. $K_2Cr_2O_7$, used as positive control, inhibited the nauplii vitality after 24 and 48 h treatment of 90 and 98%, respectively, showing a strong toxicity. No mortality or alteration of the nauplii swimming behavior were observed after the treatment with algal extracts at the concentrations range investigated, with no statistically significant differences with respect to the negative control.

3.2.2. Zebrafish embryo toxicity test

Fertilized zebrafish embryos were exposed to extracts from *A. subulata*, *H. cornuta* and *C. aerea* at two different concentrations (200 and 400 μg/ml) that resulted to be non-toxic using zebrafish FET assay. No visible toxic effects of extracts from *A. subulata, H. cornuta* and *C. aerea* at concentrations of 200 and 400 μg/ml on the development of embryos were observed (Fig. 1A). After exposing larvae to *A. subulata* at 200 μg/ml and *H. cornuta* at 400 μg/ml, only one larva (5% on total sample number) and two larvae (10% on total sample number) showed tail deformities, respectively (Fig. 1, B and C).

These data clearly suggested that crude extracts from *H. cornuta*, *A. subulata* and *C. aerea* did not affected zebrafish embryos and larval development.

3.3. Biofilm formation assay

All tested pathogenic strains were able to form biofilm with different capabilities (Table 2).

The highest biofilm production was observed for *L. anguillarum*, which displayed an OD_{585nm} value of 3.2, followed by *V. harveyi* and *P. piscicida,* while *V. parahaemolyticus* had the lowest value.

3.4. Antibiofilm activity of crude algal extracts

The effects of algal extracts at increasing concentrations (from 50 to 400 μg/ml) on biofilm formation of pathogens are reported in Fig. 2.

All the algal extracts exhibited antibiofilm activity, differing each other and also between the two red algae (*A. subulata* and *H. cornuta*) (Fig. 2). All extracts showed a dose-dependent inhibitory effects on the biofilm formation, although the antibiofilm effects depended on the strain tested. The extracts from *C. aerea* and *A. subulata* showed the strongest reduction of biofilm formation of *V. harveyi* G5 (59.1 and 52.8%, respectively) (Fig. 2A, a and b), followed by the extract from *H. cornuta* (47.0%) (Fig. 2Ac). Weak antibiofilm effects were observed for the extract from *H. cornuta* on biofilm formation by *V. parahaemolyticus* (27.8%) (Fig. 2Bc). The biofilm of *L. anguillarum* was mainly reduced by *A. subulata* (32.8%) (Fig. 2 Cb). The extracts from *A. subulata* and *H. cornuta* showed the strongest activity against the biofilm formed by *P. piscicida* (48.0% and 52.0%, respectively) (Fig. 2D, b and c).

3.5. Antimicrobial assay

All extracts at concentration from 50 to 400 μg/l did not show any activity on the bacterial growth (Table 3).

No inhibition haloes were observed using the agar diffusion assay in the presence of each extract at the highest concentration (400 μg/l) (data not shown).

3.6. Surface coating assay

All crude extracts were able to inhibit the adhesion of *V. harveyi. A. subulata* was able to prevent the adhesion of L. *anguillarum* and *P. piscicida,* whereas *H. cornuta* moderately inhibited the adhesion of *V. parahaemolyticus* and *P. piscicida* (Table 4).

3.7. Cell-surface properties and hydrophobicity

To evaluate if the algal extracts can alter the bacterial cell-surface charges and hydrophobicity, the affinity to different hydrocarbon solvents was investigated. In details, the affinity to the polar solvents, ethyl

acetate (donating electron) and to chloroform (as accepting electron), indicates the variation of charges on the cellular surfaces, whereas the affinity to the non-polar solvents, decane and hexadecane, indicates different levels of the bacterial cell-wall hydrophobicity (Bellon-Fontaine et al., 1996). The affinities to polar and non-polar solvents of *V. harveyi* G5, *V. parahaemolyticus* L12G, *L. anguillarum* Van, *P. piscicida* Pdp are reported in Table 5.

Since bacterial affinity to decane and hexadecane was low (≤50%), cellular surfaces of all bacteria were moderate or weak hydrophobic, excepting for *L. anguillarum* that showed the highest affinity to decane (>70%) (Table 5). Cellular walls of *V. harveyi* and *L. anguillarum* possessed high affinity to both chloroform and ethyl acetate, indicating the presence of both negative and positive charges on the cell-surface. *V. parahaemolyticus* showed high affinity to chloroform, indicating that its cellular surface was negatively charged. Due the weak affinity of *P. piscicida* to chloroform and ethyl acetate its cellular surfaces appeared to possess low charges.

When *V. harveyi* cells were treated with *A. subulata* extract (400 μg/ml), the affinity to ethyl acetate and decane significantly decreased (Table 5), indicating that this extract differently affected the cell- surface charges and consequently it lowered the cell-wall hydrophobicity. In the presence of *C. aerea* extract, the affinity of *V. harveyi* to chloroform decreased, while its affinity to ethyl acetate greatly increased, suggesting that the algal treatment induced a strong variation in cell-surface charges. Differently, the addition of *H. cornuta* extract increased the affinity of *V. harveyi* to hexadecane, and consequently its hydrophobicity. The extract from *H. cornuta* decreased the affinity to decane of *V. parahaemolyticus*, decreasing its hydrophobicity. *L. anguillarum* treated with *A. subulata* extract showed that the affinity to ethyl acetate, chloroform, decane and hexadecane decreased, suggesting strong variations in both cell-surface charges and hydrophobicity. In the presence of *C. aerea* extract, hydrophobicity of *L. anguillarum* decreased, while increased its affinity of ethyl acetate. *H. cornuta* extract significantly increased the affinity of *L. anguillarum* to ethyl acetate and decane. In the presence of *A. subulata* extract, the affinity of *P. piscicida* to ethyl acetate highly increased, whereas the affinity to decane and hexadecane significantly decreased. When treated with *H. cornuta* extract, the affinity of *P. piscicida* to chloroform and hexadecane decreased, indicating that its hydrophilicity increased. These results showed that the different extracts acted on the different pathogens without a general pattern, not even similarities were observed in the effects of the extracts of both the red algae.

3.8. Emulsifying activity

The emulsifying activity of algal extracts in aqueous solution (0.05%, w/v) with kerosene are reported in Table 6.

All the three algal extracts were able to form emulsions on kerosene. When compared with an equal solution of TritonX-100 in the presence of kerosene (E_{24}= 70.5 ± 0.3%), the algal extracts from *A. subulata*, *C. aerea* and *H. cornuta* possessed a good emulsion capacity, with the E_{24} values of 72.5%, 95.6% and 82.2%, respectively, and also persistent for several weeks.

4. Discussion

The bacterial biofilm formation is one of the most relevant concerns in a wide range of domains, from medicine, food industry and aquaculture, to marine environmental aspects, including biofouling. In the biofilm style of life *Vibrio* spp. and *Photobacterium* spp. enhanced their persistence in aquatic environments, being resistant to chemical disinfection and antimicrobial treatments (Nonaka et al., 2015; Barroso et al., 2019).

As previously reported, the use of natural algal extracts as potential alternatives to that of antibiotics for breeding of seabass and sea bream may represent new challenges and opportunities for fish health (Marino et al., 2016). There is a growing interest in the use of natural alternative materials to that of first-line antibiotics and biocides, particularly useful for a modern and eco-sustainable fish farming (Marino et al., 2016).

In this context and with the aim of transforming algal biomass from waste into valuable resource, in the present study the antibiofilm potential of algal crude polysaccharides from two Rhodophyta (*A. subulata* and *H. cornuta*) and one Chlorophyta (*C. aerea*) was evaluated against bacterial pathogens relevant for aquaculture.

The extracts from red algae have been reported to be constituted mainly by carrageenans, whereas from green algae by sulphated polysaccharides (Armeli Minicante et al., 2016; Lakshmi et al., 2020; Hamzaoui et al., 2020). Structurally, carrageenans and green algal polysaccharides are sulphated polysaccharides, negatively charged, with high molecular weight, with several biological properties useful as natural conservative in functional foods (Hamzaoui et al., 2020) and in human and veterinary medicine (Jiao et al., 2011; Faggio et al., 2016; Muthukumar et al., 2021). Due to the complexity of these type of polysaccharides, the attempts to establish a relationship between their structures and bioactivities/actions have not been completely elucidated.

The crude polysaccharides from *C. aerea*, *A. subulata* and *H. cornuta* showed dose-dependent inhibitory activity on the biofilm formation without exerting any antibacterial activity, and therefore confirmed a specific antibiofilm activity rather than contrasting the bacterial growth. Contextually, these data confirm previous our observations on the lack of any antimicrobial activity of crude extracts from *C. aerea* and *H. cornuta* (Rizzo et al., 2017).

Biofilm formation is a complex process, it initiates with the adhesion of bacterial cells to surfaces, which includes the initial physicochemical interaction phase, the abiotic surface characteristics and cell-surface properties and hydrophobicity, as hydrophobic cells have maximum capability to form biofilm (Katsikogianni and Missirlis, 2004; Chae et al., 2006). Based on the surface-coating assay, the algal extracts discouraged the initial cellular adhesion on polystyrene surfaces, probably due to their increased hydrophilicity in comparison to uncoated surfaces. Moreover, as emulsifiers acting at the interface between the biotic and abiotic surfaces, the algal extracts interacted with the initial adhesion of bacteria, even if at different levels.

Although taxonomically close related, each non-treated strain possessed different cell-surface properties, showing weak (*V. parahaemolyticus* L12G), moderate (*V. harveyi* G5 and *P. piscicida* Pdp) and strong (*L. anguillarum* Van) hydrophobicity, as resulted by their different affinity to non-polar solvents (decane and hexadecane) (Table 5). According to their cell surface properties, the biofilm formation on polystyrene surfaces differed among strains, with more robust biofilm formed by L. *anguillarum* and *V. harveyi*. However, only a few studies have been carried out on the hydrophobicity and adhesive ability of *Vibrio* pathogenic strains (Santos et al., 1991; Balebona et al., 2001; Acosta et al., 2021), but none study has been reported the effects of algal polysaccharides on cell-surface properties and hydrophobicity of the strains here investigated.

Consequently, the effects induced by the algal extracts on variations in cell-surface charges and cell walls hydrophobicity were investigated in relation to the biofilm formation on polystyrene surfaces (Fig. 3). Our results suggest that the antibiofilm activity of all extracts could be explained by the modification of the cell-surface charges and hydrophobicity levels. Particularly, the highest antibiofilm effect of crude extracts occurred when both these factors were modified, such as for *V. harveyi* treated with *C. aerea* and *A. subulata* extracts, as well as for L. *anguillarum* treated with *A. subulata,* and for *P. piscicida* treated with *A. subulata* or *H. cornuta*.

Our results confirm that marine algal extracts possess antiadhesive properties, useful not only in biomedical applications (Gadenne et al., 2015) but also in aquaculture.

Although cyto-toxicity of the algal extracts here studied has been previously reported (Armeli Minicante et al., 2016), studies on their *in*

vivo toxicity are lacking, including Zebrafish assays. Zebrafish is a common research model in fish studies of toxicology, biomedical research developmental biology, neurobiology and molecular genetics (Ulloa et al., 2011). All the three extracts did not show any *in vivo* toxicity effects against the two main organism models in aquaculture, *Artemia salina* and *Danio rerio,* suggesting a high eco-compatibility of the investigated algal extracts and providing useful information for their future development and applications in aquaculture.

There is well documented public concern about the use of chemicals in the aquaculture industry, in particular antibiotics and antifoulants (Costello et al., 2001; De Nys and Guenther, 2009). Natural polysaccharides, for example, alginic acid, hyaluronic acid, pectic acid and carrageenans, have been considered as alternative materials promising for marine antifouling coatings. For example, carrageenan derived from a red alga, a sulfated polysaccharide with high hydrophilicity, and low cost, was recently reported to possess marine antifouling properties (Kim and Kang, 2020; Jeong et al., 2021).

As non-biocides, non-toxic and biodegradable polysaccharides, with surfactant and antiadhesive properties, preventing hydrophobic interaction-mediated initial adherence biofilms, our extracts could be proposed to design and develop antibiofilm control measures in aquaculture. In future investigations, our extracts could be tested on the surfaces of growing facilities, such as netting material and cage netting, that represent ideal surfaces for fouling, as natural alternatives to chemical antifouling substances and paints. Moreover, they could be integrated into feeds, especially with a focus on gut health and consequent general wellbeing of fish, as well as the reduction in use of antibiotics.

Author contributions

Conceptualization, C.Gu. and V.Z.; methodology, S.G., V.Z. and M.G.R.; validation, C.Gu. and S.G.; formal analysis, S.G.; investigation, V.Z., M.G.R., A.Sp., M.S.N., G.G., M.M., D.S., F.C., C.G., A.S., D.T.; resources, C.Gu., G.G., M.M., F.C., D.T.; data curation C.Gu., V.Z. and A.Sp.; writing-original draft preparation V.Z., M.G.R. and A.Sp.; writing-review and editing, C.Gu. and S.G.; supervision, C.Gu.; funding acquisition, C.Gu.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.2 Effects of crude polysaccharides from marine macroalgae on the adhesion and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Effects of crude polysaccharides from marine macroalgae on the adhesion and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

Marine macroalgal biomass provides polysaccharides that could result in the valuable transformation of waste into an economic resource. Crude extracts, or polysaccharides, from *Chaetomorpha aerea* (Chlorophyta) and *Agardhiella subulata* and *Hypnea cornuta* (Rhodophyta), collected from the coastal brackish Lake Ganzirri (Italy), were evaluated for their effects on the adhesion and biofilm formation of *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213, as model organisms. Without exerting any antibacterial activity, each crude extract showed dose-dependent inhibitory effect on biofilm formation, differing each other and also on the strain tested. The highest concentration (400 μg mL^{-1}) of *C. aerea* extract, a sulfated polysaccharide mainly composed by glucuronic acid, strongly acted on the initial attachment of *P. aeruginosa* (54% of biofilm reduction), whereas *H. cornuta* extract greatly acted on that of *S. aureus* (59%). The addition of *A. subulata* and *C. aerea* extracts reduced the biofilm growth of *S. aureus*, but did not its mature biofilm. Conversely, *A. subulata* extract, a κ-carrageenan was also able to reduce the developed biofilm of *P. aeruginosa*. As resulted by surface-coating and the bacterial affinity to hydrocarbons assays, extracts from *C. aerea* and *H. cornuta* were able to inhibit the adhesion to polystyrene of *P. aeruginosa* and *S. aureus,* and modify their cell-surface properties and hydrophobicity. Furthermore, all the extracts formed stable emulsions. No *in vitro* cytotoxicity effects were observed for *H. cornuta* extract, while they were moderate for *C. aerea* and high for *A. subulata*. However, all the extracts did not show any potential toxicity *in vivo* fish-embryo *Danio rerio* assay. These extracts could be used to prevent or remove the bacterial biofilms in several medical and non-medical applications.

1. Introduction

Marine macroalgae or seaweeds are important sources of a great variety of compounds, such as flavonoids, tannins, polyphenols and polysaccharides, with a wide spectrum of effects useful in different biotechnological fields, including food and feed industries, cosmetic and pharmaceutical applications [1–4].

The biomass of seaweeds is largely constituted (from 4 to 76% of dry weight) by polysaccharides, such as alginates, carrageenans, agar, laminarans, fucoidans and ulvans, which are involved in important structural and storage functions. Polysaccharides are involved in cell interaction and adhesion to substrata, in contrasting the high ionic strength of seawaters [5,6], and in forming a protective barrier against pathogens [7,8]. The production of polysaccharides, also within the same algal genus, may depend on different factors, including geographical distribution, and physiological conditions [9,10]. Among them, sulfated polysaccharides (SPs) are widely distributed in varying amounts in three major divisions of seaweeds: in Rhodophyta are manly galactans; in Phaeophyta are called fucans; and in Chlorophyta are polydisperse heteropolysaccharides [11]. However, the chemical properties of such SPs, as well as their bioactivity, might be different according to their structure, molecular size, and sulfate amount [12,13]. SPs from seaweeds have been reported to possess a wide spectrum of activities, such as anticoagulant, antioxidant, antiproliferative,

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antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic, antiadhesive and antimicrobial activities [14–18].

Recently we reported that crude polysaccharides from *Chaetomorpha aerea* (Chlorophyta), *Agardhiella subulata* and *Hypnea cornuta* (Rhodophyta), collected from the coastal brackish Lake of Ganzirri (Italy), were able to inhibit biofilm formation of pathogens relevant in aquaculture (*Listonella anguillarum*, *Vibrio harveyi*, *V. parahaemolyticus* and *Photobacterium damselae* subsp. *piscicida*) [18]. Biofilm are notoriously difficult to eradicate using traditional agents or mechanical removals, since bacteria living in biofilm are less susceptibility to disinfectants and antimicrobial agents than free-living cells. Therefore, the exploration of novel strategy to prevent the initial bacterial adhesion and the biofilm growth is essential for human health, as well as in several industrial and environmental activities. The biofilm formation is a dynamic process which starts with an initial attachment to surfaces of free-living bacteria, the adhesion becomes irreversible when the cells secrete exopolymers, with the subsequent aggregation of cells and the formation and stabilization of the biofilm architecture [19]. Among the different steps in the biofilm growth, the early attachment is undoubtedly the most crucial, because different factors, including surface properties (substrate type, surface roughness and chemical composition), environmental parameters (temperature, pH, salinity, *etc.*), bacterial cell-surfaces charges and hydrophobicity, may compromise the biofilm formation and development [20,21].

The exploitation of macroalgal biomass as a source of antibiofilm molecules could result in the valuable transformation of waste into an economic resource. Invasive marine algal species present in different environments, such as that of the brackish Lake of Ganzirri (Messina, Italy), produce large amounts of biomass which needs to be periodically removed and treated as a waste [18,22,23].

In the present study the polysaccharides extracted from Chlorophyta (*C. aerea*) and two Rhodophyta (*A. subulata* and *H. cornuta*), collected from the Lake of Ganzirri, were evaluated for their effects on the adhesion and the biofilm formation of *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213, as model organisms. The crude polysaccharides were added at different times, corresponding to the different phases of biofilm formation, initial attachment (T0), reversible attachment (T4) and irreversible attachment (T8), and after the biofilm development. The Fourier-Transform Infrared spectroscopy was used to characterize the extracts. Furthermore, the effects of extracts on the bacterial cell-surface properties and hydrophobicity were discussed. The cytotoxicity of the algal extracts was investigated both *in vitro*, on BJ-5ta human foreskin fibroblast cell-line, and *in vivo* using *Danio rerio* embryos.

2. Materials and methods

2.1. Sample collection, DNA barcoding and algal extracts

Samples of Chlorophyta and Rhodophyta were collected from the brackish Lake of Ganzirri in the Cape Peloro lagoon of Messina (Italy). Fresh samples were washed with sterile seawater and manually cleaned of epiphytes. From each sample, a part was dried in silica gel and stored at −20 °C for molecular identification, and the rest was oven-dried at 40 °C for 48 h for the polysaccharide extraction.

Taxonomic characterization was performed by DNA barcoding, using COI-5P for *A. subulata* and *H. cornuta,* and LSU D2/D3 for *C. aerea,* according to Manghisi et al. [24], by an external company (Macrogen Europe, The Netherlands). Sequence reads were assembled by the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and deposited to the Barcode of Life Data Systems (BOLD systems) (http://www.boldsystems.org/).

Polysaccharides were extracted from dried samples (100 g) of macroalgae as detailed in Armeli Minicante et al. [9] with some modifications. Dried red algae were soaked in NaOH 80 g L^{-1} (about 1:1 w/v) at 70 °C in oven for 2 h, then filtered through cotton-net and washed in distilled water. Polysaccharides were extracted from thalli by soaking in distilled water (about 1:10 w/v) at 70 °C for 24 h. After removing exhausted thalli, polysaccharides were precipitated adding an equal volume of absolute ethanol and then recovered by centrifugation at 4000 rpm. Crude polysaccharide extracts were dried at 40 °C overnight. For *C. aerea*, the extraction was performed in HCl solution (pH 2) at 70 °C for 3 h, without pre-treatment in NaOH.

2.2. Antibiofilm activity of algal extracts against Pseudomonas aeruginosa and Staphylococcus aureus

2.2.1. Effects of crude polysaccharide extracts at increasing concentrations

P. aeruginosa ATCC 27853 was cultured into Luria Bertani broth (LB, Sigma Aldrich) and 2% agarized LB (LA). *S. aureus* ATCC 29213 was grown in Tryptone Soy Broth (TSB, Sigma Aldrich) and Tryptone Soy Agar (TSA, Sigma Aldrich). Strains were kept frozen at −80 °C in 40% (v/v) glycerol for long term storage. *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were purchased from the American Type Culture Collection (LGC Promochem, Milan, Italy).

The antibiofilm activity of algal extracts against *P. aeruginosa* and *S. aureus* was evaluated in 96-well polystyrene microplates (Falcon®, Fisher Scientific, Milan, Italy), as previously reported by O'Toole et al. [25].

Overnight cultures (180 μL) of *P. aeruginosa* grown in LB or *S. aureus* in TSB (6 replicates) (OD600nm = 0.1 equivalent to 1.5×10^8 bacteria mL^{-1}) were poured in the microwells and each crude polysaccharide extract (20 μL) diluted in Phosphate Buffer Saline (PBS, Sigma Aldrich) at different concentrations (50, 100, 200 or 400 μg mL^{-1}), or 20 μL of PBS as control, were added to each well. Microplates were incubated at 37 °C for 48 h (for *P. aeruginosa*) or 24 h (for *S. aureus*), without shaking. Nonadherent bacteria were removed by washing 5 times with distilled water. Biofilms were stained with 0.1% (w/v) crystal violet solution for 20 min. Excess stain was removed by aspiration, and then the plates were washed (5 times) and air dried (for 45 min). The stained biofilms were solubilized with absolute ethanol and the biofilm mass was spectrophotometrically determined (OD585nm) by the level of the crystal violet present in the de-staining solution, using a microtiter plate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA).

The reduction of biofilm formation of each strain was expressed as antibiofilm activity (%) by applying the following formula:

$$\frac{OD_{585nm}\text{Control} - OD_{585nm}\text{Sample}}{OD_{585nm}\text{Control}} * 100$$

Each data point was averaged from eight replicated microwells, and the standard deviation (SD) was calculated.

2.2.2. Effects of algal extracts on the biofilm formation

To evaluate the ability to interfere on the biofilm formation of *P. aeruginosa* and *S. aureus*, each crude polysaccharide with the highest antibiofilm activity (400 μg mL^{-1} in PBS) was added at different times (0, 4 and 8 h) and after 24 h for *S. aureus* or 48 h for *P. aeruginosa,* when the biofilm was completely established. Aliquots (180 μL) of overnight bacterial cultures (OD600nm = 0.1, equivalent to 1.5×10^8 bacteria mL^{-1}) incubated at 37 °C were poured into 96-well polystyrene microtiter plates (Falcon) and 20 μL of each extract solution or PBS (control) were added at different times. The biofilm quantification was performed using the crystal-violet staining technique, according to the method described above.

2.3. Antibacterial activity of algal extracts

The effects of each algal extract on the bacterial growth were spectrophotometrically determined. Aliquots (180 μL) (OD600nm = 0.1) from each overnight strain culture, *P. aeruginosa* and *S. aureus* (in LB or TSB, respectively), were distributed in 96-well polystyrene microtiter plates (six replicates), and 20 μL of each algal extract (400 μg mL^{-1} final

concentration in PBS) or PBS used as control, were added to each well. The microplates were incubated at 37 °C for 24 h without shaking and OD600nm values were registered.

Moreover, the antibacterial activity was evaluated by using the standard disk diffusion method (Kirby Bauer test), as accepted by the National Committee for Clinical Laboratory Standard (NCCLS 2000). *P. aeruginosa* and *S. aureus* were overnight grown (onto LA or TSA plates and incubated at 37 °C for 24 h, respectively) and colonies from each strain were suspended in 3 mL of 0.9% NaCl solution (OD600nm = 0.1). Suspensions were inoculated in triplicate onto plates of Mueller Hinton agar (Oxoid) using a cotton swab.

Each algal extract (20 mg) was dissolved in 1000 μL of sterile distilled water and 20 μL of each solution were applied to sterile filter paper disks (6 mm in diameter, Oxoid). After evaporation, the disks (containing each 400 μg of extract) were placed onto inoculated plates. Plates were incubated overnight at 37 °C. The diameter of inhibition zone was measured and means and standard deviations (n= 3) were calculated.

2.4. Surface coating assay

A volume of 20 μL of each extract diluted in PBS (400 μg mL^{-1} final concentration), or 20 μL of PBS used as control, were transferred to the centre of 24-well polystyrene microtiter plates (Falcon no. 353047) [26]. The plates were incubated for 30 min at 37 °C to allow the complete evaporation of the liquid. Each well was filled with 1 mL of diluted overnight bacterial culture (containing 10^5 CFU mL^{-1}) in LB for *P. aeruginosa* or in TSB for *S. aureus*. After incubation at 37 °C for 18 h in static conditions, the wells were emptied gently, washed with distilled water and stained with 1 mL of 0.1% crystal violet solution. To remove the excess of crystal violet, stained biofilms were rinsed with distilled water and air dried.

2.5. Bacterial cell-surface properties and hydrophobicity

To analyse the bacterial cell-surface properties, the microbial adhesion to hydrocarbons test (MATH) was used according to Bellon-Fontaine et al. [27]. *P. aeruginosa and S. aureus* cells grown overnight (in LB or TSB, respectively) at 37 °C, were harvested by centrifugation (6000 rpm for 10 min) and washed twice with PBS. Each bacterial suspension (2 mL) was treated for 30 min with each algal extract solution (400 μg mL^{-1} in PBS), or with PBS as control. Treated and not-treated bacterial suspensions were centrifuged (6000 rpm for 10 min) and resuspended in PBS with OD400nm comprised from 0.5 to 0.7 (A0). Aliquots (3 mL) of each treated and not-treated bacterial suspension were added to each tube containing 0.4 mL of the following hydrophobic solvents (Sigma-Aldrich): ethyl acetate, a strong basic solvent; chloroform, an acidic solvent which exhibits negligible basic character; decane, as a non-polar solvent having intermolecular attraction comparable to that of ethyl acetate, and hexadecane, as a non-polar solvent having intermolecular attraction comparable to that of chloroform. After vigorous agitation by vortex, phases were allowed to separate for 10 min at 30 °C and the OD400nm of the aqueous phase was measured (A1). The percentage of affinity to hydrocarbons was calculated as follows:

$$\%\text{Affinity} = \frac{A0 - A1}{A0} * 100$$

2.6. Emulsifying activity

To evaluate the emulsifying activity of the crude algal extracts, the method described by Mata et al. [28] was used. Each crude lyophilised extract, dissolved in 2 mL distilled water (0.05%, w/v), was mixed with an equal volume of kerosene in a glass tube (10 cm high and 1 cm in diameter) and vortexed at high speed for 2 min. The emulsification index (E_{24}) was calculated by the following formula [29]:

$$E_{24} = \frac{\text{High of the emulsion layer}}{\text{Total high of the mixture}} \times 100$$

Triton X-100 (Sigma-Aldrich) was used as positive control.

2.7. Characterization of algal extracts

2.7.1. Fourier-Transform Infrared Spectroscopy (FT-IR) analysis

The Fourier-Transform Infrared Spectroscopy (FT-IR) spectra of dried extracted polysaccharide standards were recorded by Nicolet iS 5 Thermo Scientific equipped with iD5 ATR. The spectra were scanned at room temperature in the range 4000–600 cm^{-1}, with a resolution of 4 cm^{-1} using a diamond crystal. Triplicates of each sample were averaged to obtain an average spectrum. A background spectrum of air was scanned under the same instrumental conditions before each measurement series.

2.7.2. Gel permeation chromatography

The molecular weight of the polysaccharides was detected by gel permeation chromatography (Agilent GPC PL-GPC 220) equipped with a refractive index detector (cell volume 8 μL, wavelength 890 nm). For polysaccharides from *C. aerea*, the following conditions were used: sodium nitrate 0.1 M as mobile phase, 1 mL min^{-1} flow rate, 30 °C column temperature and 100 μL injection volume of 5 mg mL^{-1} sample aqueous solution. For polysaccharides from red algae, the same condition was used with slightly modification as follows: sodium nitrate 0.05 M as mobile phase, column temperature was set at 35 °C. For polysaccharides the calibration was performed using six molecular weights of pullulan standards in the range 350–700 kDa.

2.7.3. Monosaccharide composition of the green seaweed extract

The monosaccharides composition of the extracted polysaccharides was evaluated by gas chromatography (Agilent technologies HP 6890) equipped with a flame ionization detector (FID) and J&W HP1 capillary column. The following set-up was used: injector temperature 250 °C and detector temperature 280 °C. The oven temperature was increased from 120 to 260 °C at a rate of 5 °C min^{-1}. Nitrogen has been used as carrier gas at a flow rate of 0.9 mL min^{-1}. The assay was performed according to the following protocol. Dried polysaccharide (50 mg) was hydrolyzed using sulfuric acid (1 M) for 3 h at 100 °C. Afterwards, the solution was cooled, neutralized with ammonium hydroxide 15 M and 400 μL mixed with 2 mL of sodium borohydride solution in DMSO. The mixture was left for 90 min at 40 °C in a water bath. Afterwards, 0.5 mL, 0.4 mL and 4 mL of glacial acetic acid, 1-methylimidazole and acetic anhydride were added, respectively. The mixture was cooled and then distilled water (10 mL) and dichloromethane (3 mL) were added, and the lower phase was recovered and used for the analysis.

2.8. Cytotoxicity assay

For cell biology tests, human foreskin fibroblast BJ-5ta cell line (ATCC, catalog#CRL-4001) was used, because dermal fibroblasts have a crucial role in cell biology study concerning healing of wounds, as they contribute to generating connective tissue that imparts elasticity to the epidermis. BJ-5ta cells were cultured in DMEM medium (Sigma-Aldrich) supplemented with 2 mM L-Glutamine (Euroclone, Italy), 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin/amphotericin (PSA) (Euroclone). Cells were maintained in a humidified environment at 37 °C and 5% CO_2/95% air atmosphere. The medium was replaced twice a week and cells were split at about 80% of confluence.

To evaluate the cytotoxicity of algal extracts at several concentration (100, 200 and 400 μg mL^{-1}) we performed a 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Italy) assay

as previously reported [30,31]. Specifically, 24 and 48 h after cell incubation with algal extracts, the medium from each well was removed, replaced with FBS free medium containing MTT (1 mg mL^{-1}) and incubated for 2 h at 37 °C and 5% CO2. After removing MTT solution, each well was washed 2 times using cold PBS 0.01 M, and the formed crystals were melted using DMSO. To quantify the cell viability the absorbance at 570 nm was read using a synergy HT plate reader (BioTek Instruments, Inc., VT, United States). Cell viability values were expressed as percentage ± standard deviation (SD). The data were obtained from three independent determinations.

2.9. Toxicity of algal extracts on in vivo Danio rerio embryos

The Zebrafish Embryo Acute Toxicity Test (ZFET) was conducted at the Centre for Experimental Fish Pathology (CISS), located at the Department of Veterinary Sciences, University of Messina, accredited for the use and production of aquatic organisms for experimental research. All procedures were performed according Directive 2010/63/EU. Adults *Danio rerio* were reared in a ZebTEC Stand Alone system (Tecniplast). Animals were maintained at 27.5 ± 1 °C, pH 7.3 ± 0.3, conductivity 650 ± 50 mS cm^{-1}, dissolved oxygen content (DO) of 7.00 ppm and 14/10 h dark/light regimen. *D. rerio* were fed twice daily with Gemma micro 300 (Skretting, Varese, Italia) and *Artemia nauplii* (Artemia Cyst, Blue Line, Italy).

Adult zebrafish were set up in spawning tanks and after mating, the eggs were collected and bleached as reported by Westerfield [32]. Fertilized eggs were used for ZFET. To estimate the toxicity of the three algal extracts on zebrafish embryo, the Fish Embryo Acute Toxicity (FET) was carried out according to OECD guideline [33]. After bleaching, eggs were placed into 24-well plates (LABSOLUTE, Th.Geyer GmbH & Co.KG) (1 embryo per well) in 3 mL solution/well.

According to OECD guideline, the toxicity was estimated by several endpoint: coagulation of the embryo, lack of somites formation, non-detachment of the tail, non-detection of the heartbeat and number of hatched embryos were recorded. At the end of the exposure period (96 hpf), the lethal concentration (LC_{50}) was calculated. Algal extracts from *C. aerea, A. subulata* and *H. cornuta* were dissolved in Embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$; pH 7.3) reaching the final concentrations of 200 μg mL^{-1} and 400 μg mL^{-1} for each extract. Control group was kept in stock embryo medium.

All the observations were made at 24, 48, 72, 96 hpf under stereoscope (Leica M205 C). Embryos were incubated at 26 °C for 96 hpf. All extract solutions and embryo medium were renewed daily at 90% after recording any toxic or lethal effects on the embryos.

2.10. Statistical analysis

Statistical analysis was performed by One-way ANOVA followed by Holm *post hoc* test. Differences were considered statistically significant for *p*- value of <0.01 or 0.05.

3. Results

3.1. Algal identification

The algal tested were identified, and DNA barcode sequences were submitted to the BOLD System to allow genetically labelled (Table 1).

Table 1
Marine macroalgae used in this study, identification and BOLD accession numbers.

Phylum	Species	Extract ID	BOLD accession number
Chlorophyta	*Chaetomorpha aerea*	POL004	GRAPP001-17
Rhodophyta	*Agardhiella subulata*	POL046	ITRED008-10
Rhodophyta	*Hypnea cornuta*	POL045	ITRED081-13

3.2. Antibiofilm activity of crude algal extracts

Biofilm formation of *P. aeruginosa,* grown in LB after incubation at 37 °C for 48 h, displayed an OD585nm value of 2.1, whereas that of *S. aureus,* grown in TSB at 37 °C for 24 h, was 1.1.

3.2.1. Effects of extracts at increasing concentrations

The effects of algal extracts at increasing concentrations (from 50 to 400 μg mL^{-1}) on biofilm formation of the two strains are reported in Fig. 1.

All the algal extracts showed a dose-dependent inhibitory effect on the biofilm formation of *P. aeruginosa* and *S. aureus* (Fig. 1A and B). At the highest concentration (400 μg mL^{-1}), the extracts from *C. aerea* and *A. subulata* showed the strongest reduction of biofilm formation of *P. aeruginosa* (53.6% and 44.5%, respectively) (Fig. 1A, a and b), whereas a weaker effect was observed for the *H. cornuta* extract (24.7%) (Fig. 1Ac).

The extracts from *A. subulata* and *H. cornuta* (400 μg mL^{-1}) showed the strongest activity against the biofilm formed by *S. aureus* (51.6% and 59.0%, respectively) (Fig. 1B, b and c), whereas the *C. aerea* extract exhibited a weaker effect (25.4%) (Fig. 1Ba).

3.2.2. Effects of algal extracts on the biofilm formation

The effects of the addition of extracts from *C. aerea*, *A. subulata* and *H. cornuta* at the highest concentration (400 μg mL^{-1}) on the biofilm formation at different times (0, 4 and 8 h), and after 24 h for *S. aureus* or 48 h for *P. aeruginosa,* when the biofilm was completely established, are showed in Fig. 2.

Each algal extract appeared to have a different mode of action on the *P. aeruginosa* and *S. aureus* biofilm formation. The addition of extracts from *C. aerea* and *H. cornuta* inhibited significantly the initial attachment (T0) and reversible attachment (T4) of *P. aeruginosa* (Fig. 2 Aa and Ac). Differently, the addition of the *A. subulata* extract was able to inhibit the irreversible attachment (T8 = 30.1%) and also to destabilize the mature biofilm of *P. aeruginosa* (T48 = 30.1%) (Fig. 2b).

None of the extracts was able to reduce the mature biofilm (T24) of *S. aureus* (Fig. 2B). However, the extracts of *C. aerea* and *A. subulata* reduced both the reversible attachment (25.5 and 27.2%, respectively) and the irreversible attachment (25.0 and 27.1%, respectively) of *S. aureus*. The addition of *H. cornuta* extract inhibited significantly only the reversible attachment (21.3%) of *S. aureus*.

3.3. Antimicrobial activity of the extracts

All extracts at increasing concentrations (from 50 to 400 μg mL^{-1}) did not significantly affect the bacterial growth (Table S1). No inhibition haloes were observed using the agar diffusion assay in the presence of each extract at the highest concentration (400 μg mL^{-1}).

3.4. Surface coating assay

All crude extracts interfered with the adhesion on polystyrene surfaces of *P. aeruginosa* and *S. aureus,* with different capability (Table S2). *C. aerea* strongly contrasted the cells adhesion of both strains, whereas *H. cornuta* was more effective in preventing the adhesion of *P. aeruginosa* than *S. aureus*. *A. subulata* inhibited moderately the cellular adhesion of both strains.

3.5. Cell surface and hydrophobicity properties

The affinity to different hydrocarbon solvents (MATH) was investigated in order to evaluate the effects of algal extracts on bacterial cell-surface charges and hydrophobicity. In details, the affinity to the polar solvents, ethyl acetate (as donating electron) and chloroform (as

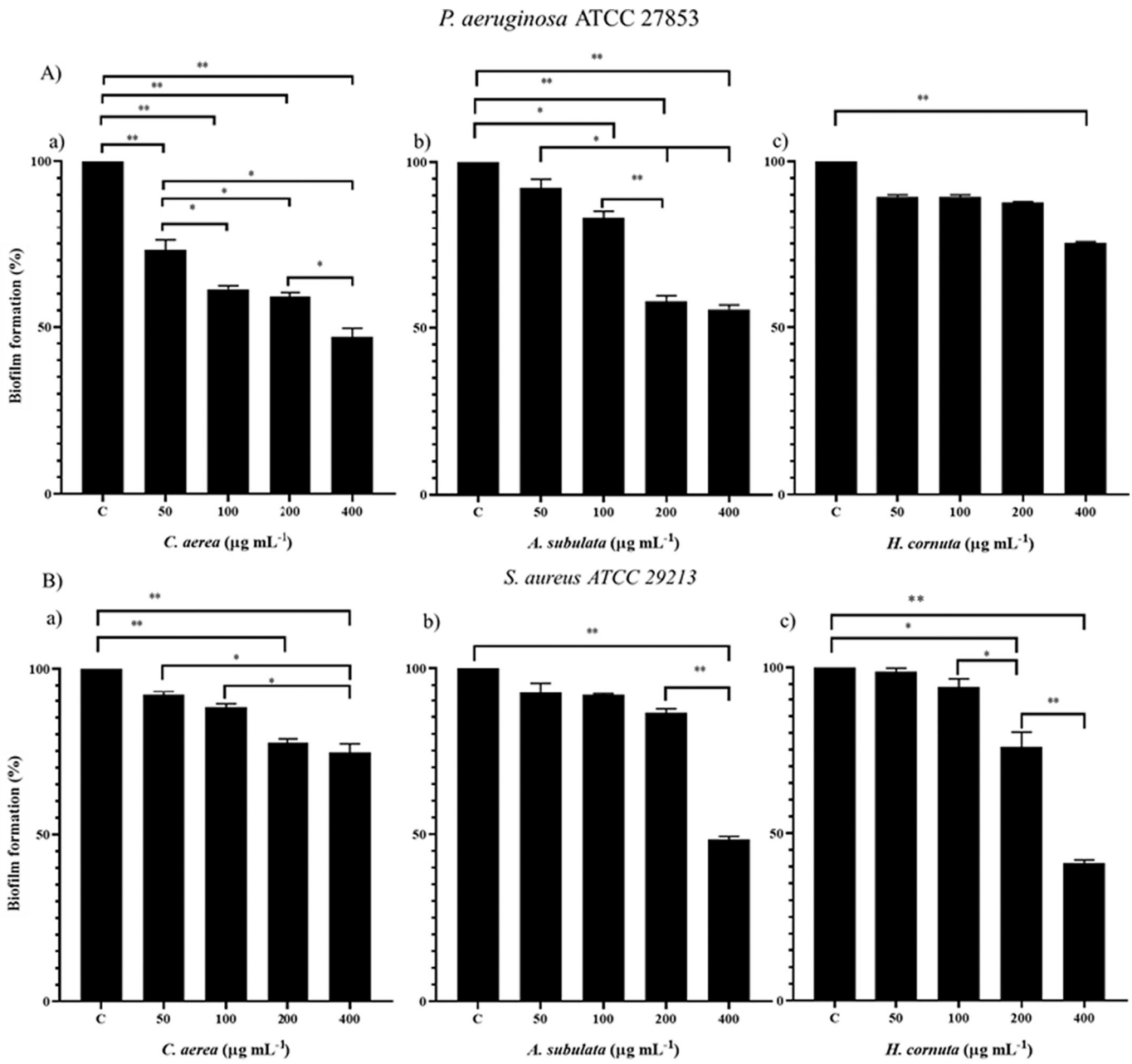

Fig. 1. Biofilm formation (%) by *Pseudomonas aeruginosa* ATCC 27853 (A) and *Staphylococcus aureus* ATCC 29213 (B) in absence (control, C) or in the presence of the crude extract from *Chaetomorpha aerea* (a), *Agardhiella subulata* (b) and *Hypnea cornuta* (c) at increasing concentrations (from 50 to 400 µg mL^{-1}). Data represent mean ± SD for six replicates (n = 6). Significantly different ** $p < 0.01$ or *$p < 0.05$.

accepting electron), indicates the variation of charges on the cellular surfaces, whereas the affinity to the non-polar solvents, decane and hexadecane, indicates different levels of the bacterial cell-wall hydrophobicity [29]. The affinities to polar (ethyl acetate and chloroform) and non-polar solvents (decane and hexadecane) of *P. aeruginosa* and *S. aureus* are showed in Fig. 3.

Untreated *P. aeruginosa* possessed high affinity to chloroform (61.9%), low affinity (<50%) to ethyl acetate, decane and hexadecane, indicating that cellular surfaces were negatively charged and moderate hydrophobic (Fig. 3A). Not-treated *S. aureus* cells showed high affinity (88.1%) to chloroform and low (20.2%) to ethyl acetate, whereas the affinity to decane and hexadecane was high (51.7 and 52.3% respectively), suggesting that cellular surface was negatively charged and hydrophobic (Fig. 3B).

C. aerea extract (400 µg mL^{-1}) significantly modified the affinity to all the hydrocarbons tested of *P. aeruginosa* (Fig. 3Aa). In detail, the affinity to ethyl acetate increased, whereas the affinity to chloroform, decane and hexadecane decreased, indicating that this extract affected both the cell surface charges and the cell-wall hydrophobicity.

Differently, the addition of *A. subulata* extract decreased the affinity of *P. aeruginosa* to non-polar solvents (decane and hexadecane), decreasing its hydrophobicity (Fig. 3Ab). In the presence of *H. cornuta* extract, only the affinity to chloroform of *P. aeruginosa* decreased, suggesting that this treatment induced a moderate variation in cell-surface charges (Fig. 3Ac).

In the presence of *C. aerea* extract, the affinity to ethyl acetate and chloroform of *S. aureus* decreased, suggesting a variation in the cell-surface charges (Fig. 3Ba). *A. subulata* extract significantly modified the affinity to all the hydrocarbons of *S. aureus* (Fig. 3Bb), indicating that this extract affected both the cell-surface charges and the cell-wall

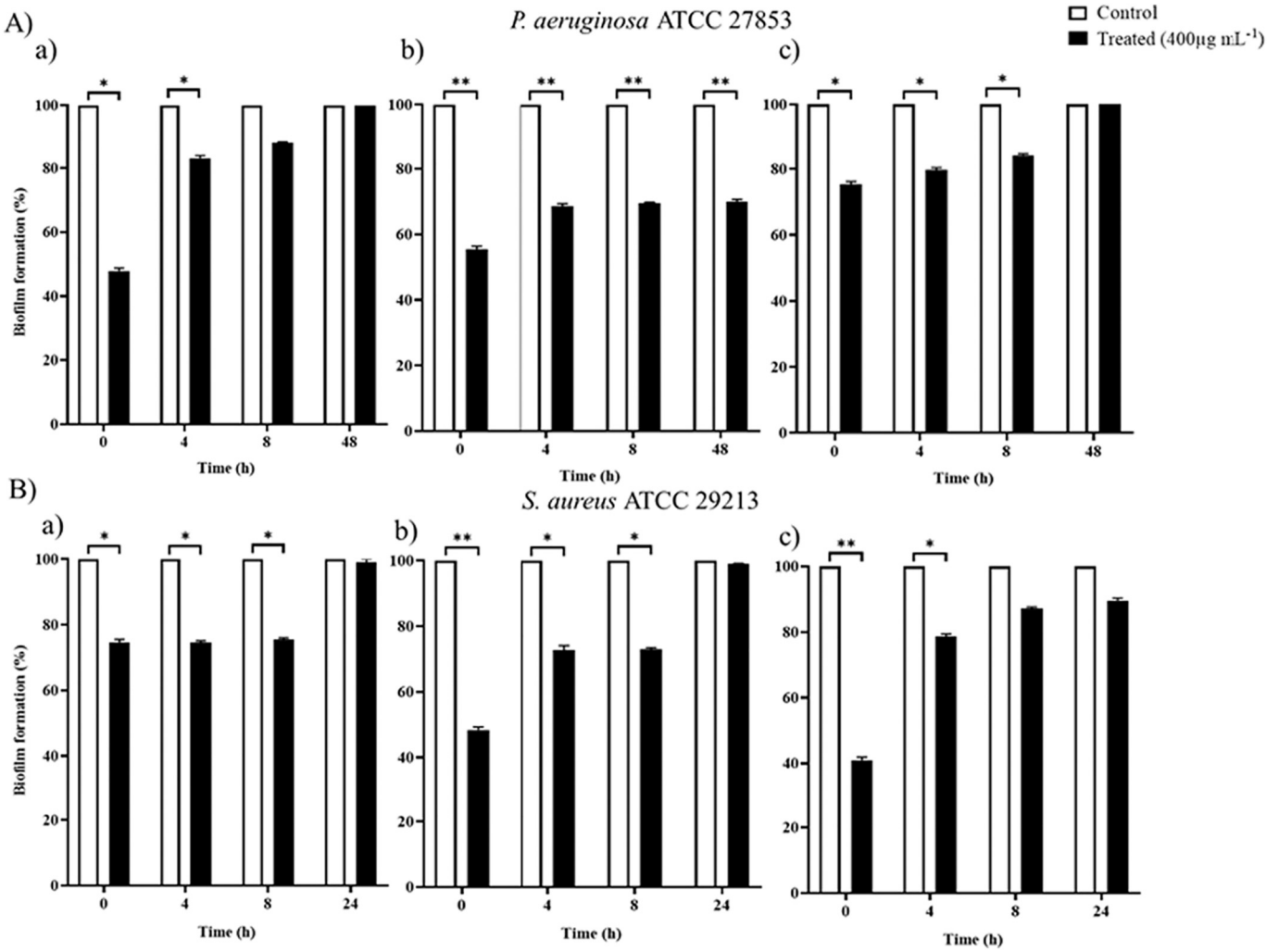

Fig. 2. Biofilm formation (%) by *Pseudomonas aeruginosa* ATCC 27853 (A) and *Staphilococcus aureus* ATCC 29213 (B) in absence (control, C) or after the addition of the crude extracts (400 µg mL^{-1}) from *Chaetomorpha aerea* (a), *Agardhiella subulata* (b), and *Hypnea cornuta* (c) at different times (T0, T4, T8), and after 48 h (T48) for *P. aeruginosa* and 24 h (T24) for *S. aureus* when the biofilms were completely established. Significantly different ** $p < 0.01$ or *$p < 0.05$ compared with untreated control (white bar).

hydrophobicity. *H. cornuta* significantly decreased the affinity to decane and hexadecane of *S. aureus*, lowering the cell wall hydrophobicity (Fig. 3Bc).

3.6. Emulsifying activity of algal extracts

The emulsifying activity of algal extracts in aqueous solution (0.05%, w/v) with kerosene is reported in Table 2.

When compared with an equal solution of TritonX-100 in the presence of kerosene (E_{24}= 70.5 ± 0.3%), the algal extracts from *A. subulata*, *C. aerea* and *H. cornuta* possessed a good emulsion capacity, with the E_{24} values of 72.5%, 95.6% and 82.2%, respectively. Emulsions were persistent for several weeks.

3.7. Characterization of algal extracts

3.7.1. Fourier-Transform Infrared Spectroscopy

The FTIR spectra of *A. subulata*, *C. aerea* and *H. cornuta* extracts are reported in Fig. 4.

In the spectrum obtained for *C. aerea* extract (Fig. 4A), the signal at 1620 cm^{-1} is attributed to the asymmetric stretching of the carboxylic groups in the uronic acid residues, while the peak 1230 cm^{-1} can be attributed to the stretching vibration of the S═O of the sulfate groups [34]. At 1002 cm^{-1} the intensive peak could be ascribed to the C—O stretching of the rhamnose and uronic acid [35]. The signals at 862 cm^{-1} correspond to the bending vibration of C-O-S of the sulfate in axial position. Other peaks are found at 2920 cm^{-1}, 1640 and 809 cm^{-1} which are related to the C—H stretching vibration, symmetric stretching of the carboxylic groups and the bending vibration of the C-O-S of sulfate in the equatorial position, respectively [36]. The broad band centered at 3330 cm^{-1} is related to the stretching vibration of O—H. The extract from *C. aerea* resulted a sulfated polysaccharide.

The spectra of the extracts from *H. cornuta* (track a) and *A. subulata* (track b) are reported in Fig. 4B. The peak at 1216 cm^{-1} is ascribed to the sulfate ester; at 1020 cm^{-1} is related to the glycoside bond. The peaks in the range 924–935 cm^{-1}, 839–845 cm^{-1} are related to the 3,6 anhydrogalactose and galactose-4-sulfate, respectively [37]. In both spectra, the characteristic peaks related to the carrageenan are presented, and the main differences in terms of peaks intensity are observed in the fingerprint region [38]. The most evident difference between spectra *a* and *b* is related to the intensity of the peak and in particular related to the S═O which has lower intensity for the carrageenan extracted from *A. subulata*. In addition, in the carrageenan extracted from *H. cornuta* the peak at 797 cm^{-1} which could be ascribed to the 3,6 anhydrogalactose-2-sulfate has a very low intensity in the carrageenan from *A. subulata*. The structure of the various types of carregeenans is

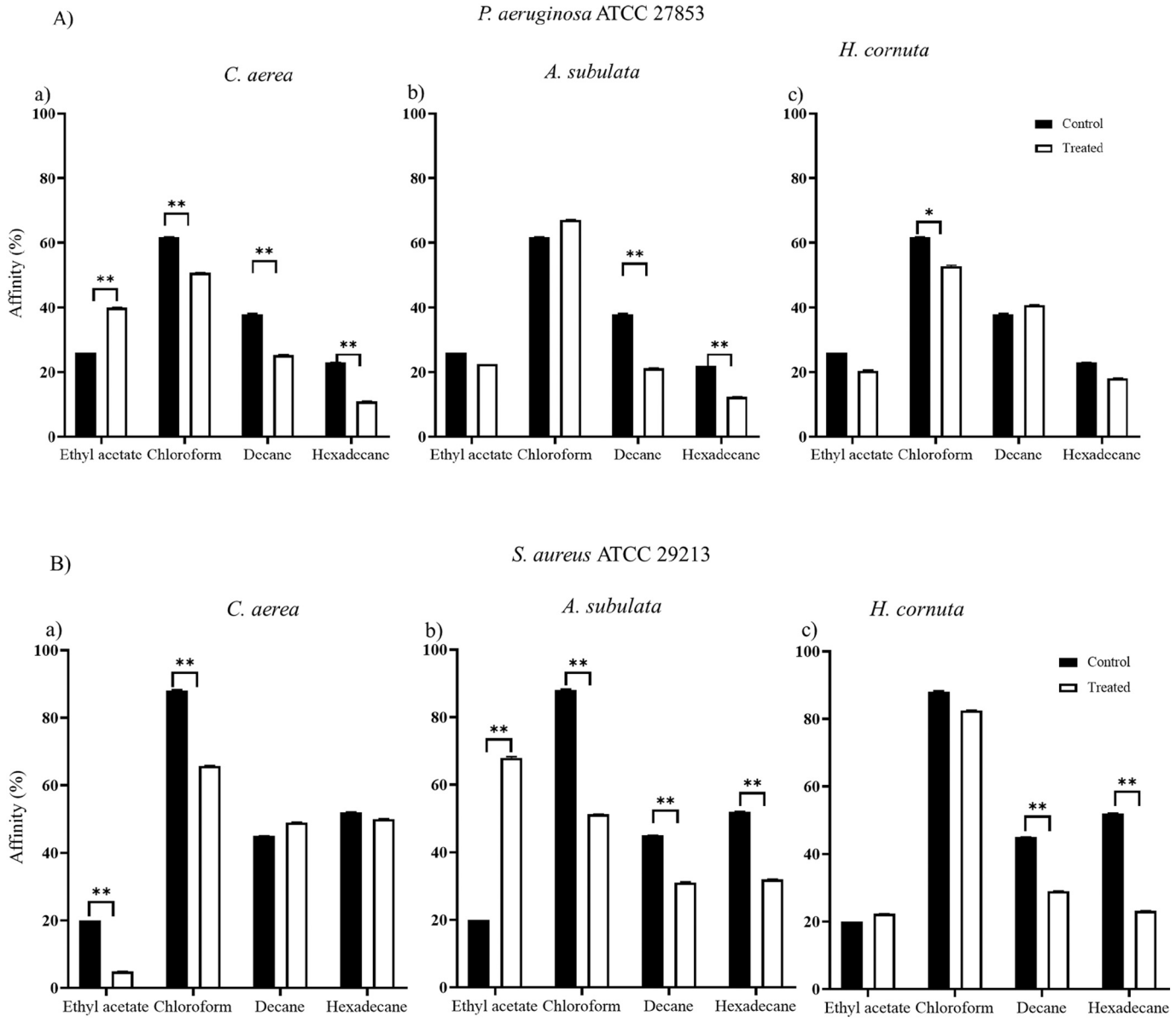

Fig. 3. Affinity (expressed as percentage) to polar (ethyl acetate and chloroform) and non-polar solvents (decane and hexadecane) of *Pseudomonas aeruginosa* ATCC 27853 (A) and *Staphylococcus aureus* ATCC 29213 (B) cells, not treated or treated with crude extracts (400 μg mL^{-1}) from *Chaetomorpha aerea* (a), *Agardhiella subulata* (b) and *Hypnea cornuta* (c). Significantly different ** $p < 0.01$ or *$p < 0.05$ compared with not-treated controls.

Table 2
Emulsifying activity of crude extracts from *Chaetomorpha aerea, Agardhiella subulata* and *Hypnea cornuta* in comparison with Triton X-100.

Extract	E_{24} (%)
Chaetomorpha aerea	95.6 ± 0.6
Agardhiella subulata	72.5 ± 0.2
Hypnea cornuta	82.2 ± 0.4
Triton X-100	70.5 ± 0.3

defined by the number and position of sulfate groups, the presence of 3,6-anhydro-d-galactose and conformation of the pyranosidic ring [37]. In the present case, the extract from *H. cornuta* seems to have a higher content of carrageenan with a higher concentration of sulfate groups like ν, μ, ι and λ, while in that from *A. subulata* a higher content of κ and β.

3.7.2. Molecular weight determination by gel permeation chromatography

The molecular weight of polysaccharides extracted from *C. aerea*, *A. subulata* and *H. cornuta* were assessed by gel permeation chromatography (Table 3).

For the polysaccharide extracted from *C. aerea* the molecular weight analysis revealed a two peaks distribution; a main peak reflecting a high Mw 1413–1920 kDa and a second smaller one representing Mw 590–730 kDa. In accordance with data reported in the literature, in which the Mw of polysaccharides in absence of degradation falls in the range from 1600 to 1900 kDa, we can conclude that the reported extraction procedure did not cause any significate degradation.

Both samples related to carrageenan possessed a high molecular weight and the Mw calculations were performed in the elution time range from 5 to 10 min. Carrageenan extracted from *H. cornuta* demonstrated a mono-distribution profile with a peak reflecting molecular weight in the range 340 to 510 kDa, while in the carrageenan extracted from *A. subulata* two peaks were detected, the major one corresponding to Mw 410–540 kDa and a minor one representing a

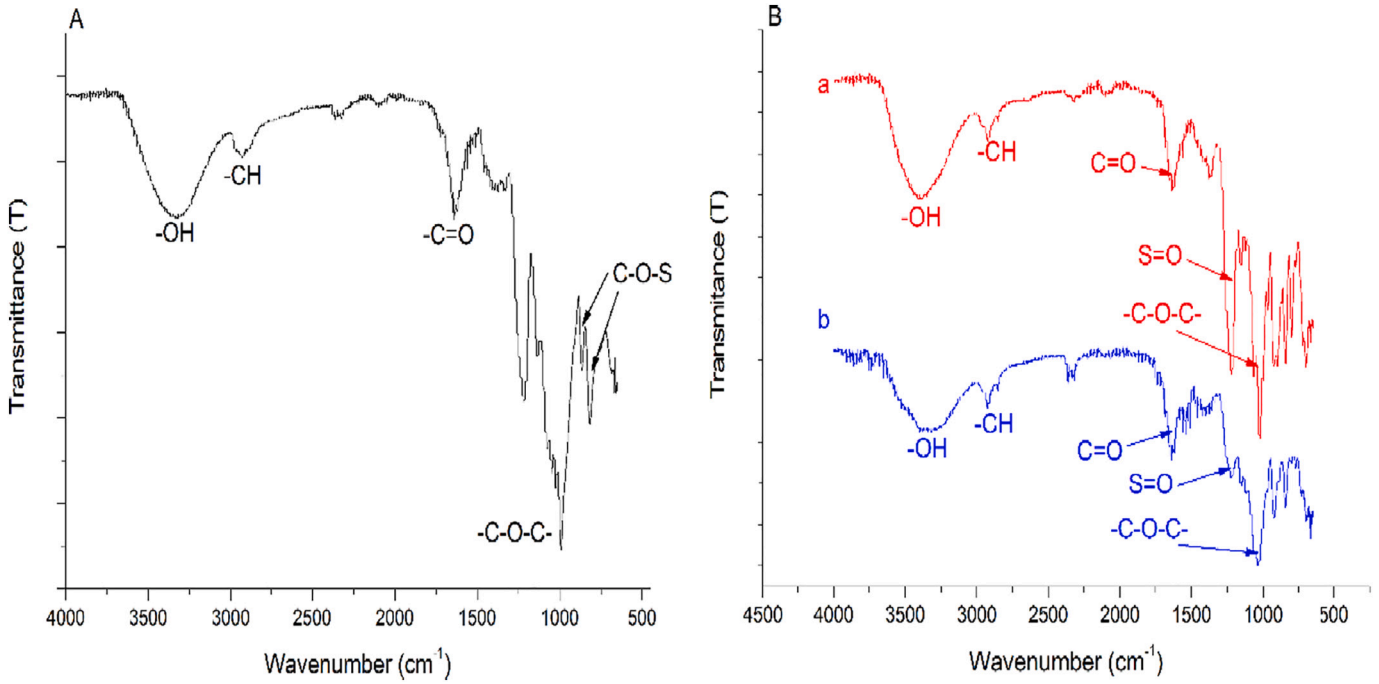

Fig. 4. FTIR spectra of extracts from the green *Chaetomorpha aerea* (A) and red algae (B), *Hypnea cornuta* (a) and *Agardhiella subulata* (b).

Table 3
Molecular weight (Mw) and refractive index (RI) of polysaccharides extracted from macroalgae.

Sample	M_{w1} (kDa)	RI_1 (mV)	M_{w2} (kDa)	RI_2 (mV)
C. aerea	1625	38	665	14
A. subulata	475	39	80	17
H. cornuta	425	41	–	–

lower molecular weight fragment between 60 and 100 kDa.

3.7.3. Monosaccharide composition of Chaetomorpha aerea extract

The main monosaccharide component of the *C. aerea* extract was guluronic acid (26%) followed by rhamnose (18%) (Table 4).

3.8. Cytotoxicity of crude extracts

The biocompatibility of the three algal extracts was evaluated *in vitro* at different concentrations (100, 200 and 400 μg mL^{-1}) on BJ-5ta cell line at two incubation times (24 and 48 h) (Fig. 5).

After 24 h of incubation, *C. aerea* extract showed a cytotoxic effect at 200 μg mL^{-1}, which became more pronounced at 400 μg mL^{-1}. *A. subulata* extract reduced significantly the cell viability at all the concentrations used, indicating a cytotoxic effect. *H. cornuta* exhibited a slight cytotoxic effect only at the highest concentration. After 48 h of treatment, *C. aerea* and *A. subulata* extracts showed cytotoxic effects at increasing concentrations. Differently, *H. cornuta* extract did not exhibit any cytotoxic effects.

Table 4
Monosaccharide composition, expressed in percentage of the dried weight, of the polysaccharide extract from *Chaetomorpha aerea*.

Monosaccharide	Sugar monomers content (%)
Glucuronic acid	26.3 ± 0.4
Rhamnose	18.4 ± 0.7
Xylose	8.6 ± 0.5
Iduronic acid	4.8 ± 0.2
Glucose	3.9 ± 0.5
Mannose	0.7 ± 0.1
Arabinose	0.3 ± 0.1

3.9. Toxicity of algal extract in vivo Danio rerio embryos

Zebrafish embryos exposed to algal extracts of *C. aerea*, *A. subulata* and *H. cornuta* at different concentrations (200 and 400 μg mL^{-1}) did not show any significative toxic effects (Fig. 6). After the exposure to algal extract up to the 96 hpf, no mortality was observed for both treated groups and control groups. In addition, control and experimental groups hatched normally at 48 hpf. After the exposure period, there were no significant effects, such as malformation during embryonic development, hatching delay and heartbeat alteration compared to the control group. Moreover, at 96 hpf, only 1 larva exposed to *A. subulata* at 200 μg mL^{-1} and two larvae exposed to *H. cornuta* at 400 μg mL^{-1} showed tail deformities (Fig. 6). All the OECD endpoint previously described were not observed, and based on these results we can confirm that extracts from *C. aerea*, *A. subulata* and *H. cornuta* and are not toxic for zebrafish embryos.

These data clearly suggested that crude extracts from *C. aerea, A. subulata* and *H. cornuta* did not affected zebrafish embryos and larval development.

4. Discussion

Microbial biofilms pose relevant problems in a wide range of areas, from medicine and food industries to environmental aspects. As bioactive compounds, polysaccharides from marine algae have been proved to be promising molecules for several bioprospecting purposes, including nutraceutical, functional food, cosmetic and pharmaceutical applications [12]. Several molecules, including polysaccharides, isolated from green, brown and red algae have been reported to possess potent antimicrobial activity [10]. However, most biocides act effectively on free-living bacterial cells, but are seldom useful against bacteria in biofilm lifestyle. Therefore, exploring novel and cheaper natural compounds, with less side effects than chemical and antibiotic agents, could provide novel approaches to effectively prevent the bacterial adhesion and biofilm formation.

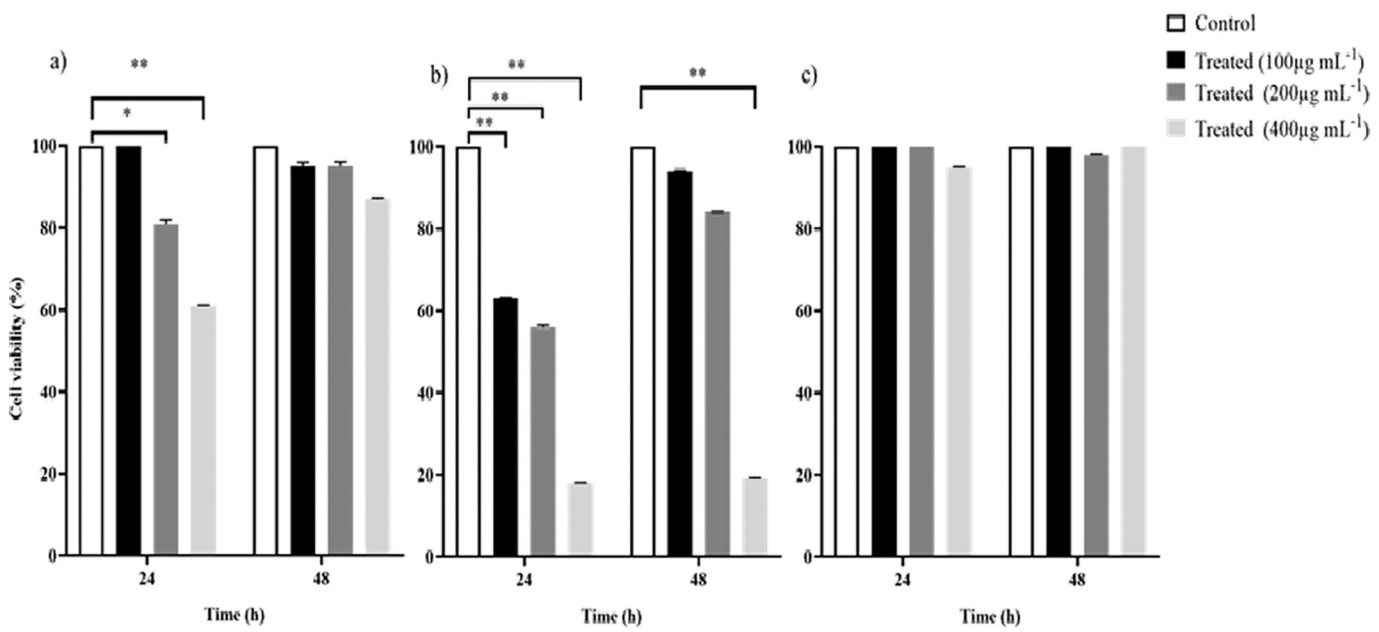

Fig. 5. Cytotoxic effects of *Chaetomorpha aerea* (a), *Agardhiella subulata* (b) and *Hypnea cornuta* (c) on BJ-5ta cells after 24 and 48 h of incubation. Significantly different ** $p < 0.01$ or *$p < 0.05$ compared with not-treated controls (C).

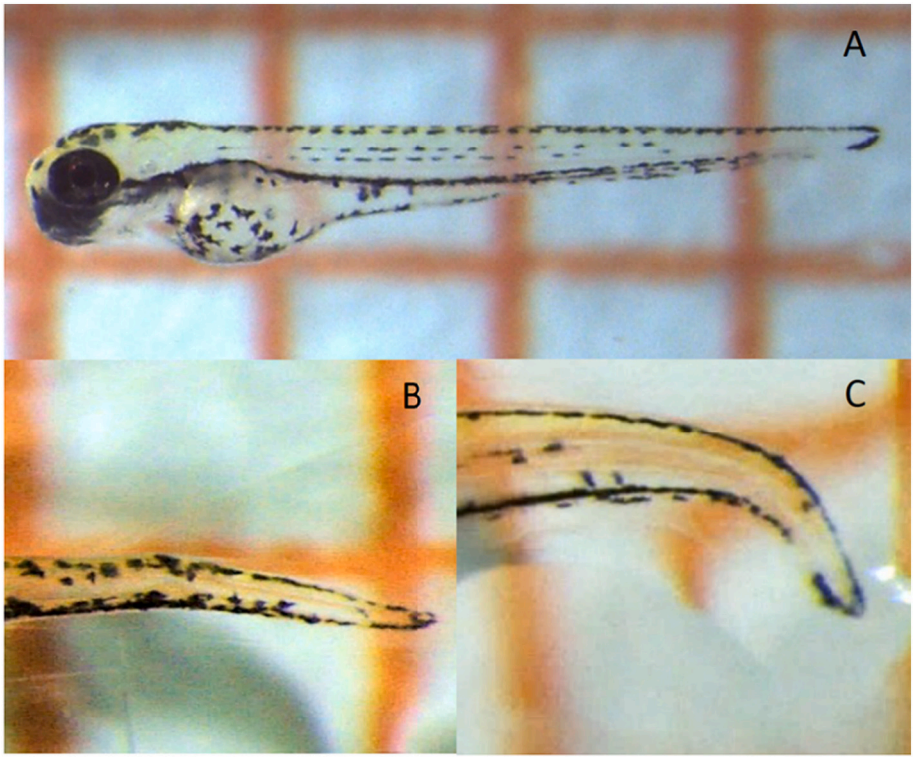

Fig. 6. Zebrafish larvae at 96 h; Control group (A); *Hypnea cornuta* extract (400 µg mL^{-1}) induced tail deformities: Zebrafish larvae with scoliosis of tail (B) and Zebrafish larvae with hooked tail (C).

In this paper the attention was focused on polysaccharides extracted from *C. aerea*, *A. subulata* and *H. cornuta*, collected from a brackish environment, as antibiofilm molecules able to contrast the biofilm formation of *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213. To evaluate whether the reduction of the biofilm formation by algal extracts was due to the inhibition of the initial attachment or in the subsequent phases of biofilm formation, the algal extracts were added at different times, corresponding to the following biofilm phases: initial adhesion, reversible attachment, irreversible attachment and mature developed biofilm of *P. aeruginosa* and *S. aureus*.

Each crude extract or polysaccharide showed dose-dependent inhibitory effect on biofilm formation, without exerting any antibacterial activity, indicating that the extracts acted specifically as antibiofilm agents. The antibiofilm activity of the algal extracts differed among the tested species, since *C. aerea* extract strongly acted on the initial attachment of *P. aeruginosa,* similarly to *H. cornuta* extract on *S. aureus,*

indicating a different action against Gram-negative and Gram-positive bacteria, that are known to produce biofilms with different features [39,40]. Moreover, the extract from *A. subulata* was the only extract able to disassemble the preformed biofilm of *P. aeruginosa.*

The initial bacterial attachment to surfaces and biofilm formation are subjected to different factors, such as physical and chemical properties of biotic or abiotic adhesion surfaces, and cell-wall charges and hydrophobicity properties. The surface coating assay confirmed that all the algal extracts greatly contrasted the bacterial adhesion on polystyrene surfaces, although with some differences. Polysaccharides showed also high emulsifying properties, being over than 70%, suggesting that they acted as biosurfactants that generally alter the surface properties (wettability and charges), and therefore, decrease bacteria-surface interactions [41,42].

On the other hand, it is well known that the activity of algal polysaccharides depends on chemical properties, such as structure, molecular size, and sulfate amount. On the basis of the spectroscopic investigation and chemical composition, the algal extracts were ascribed to sulfated polysaccharides that possess significant structural differences regarding the monosaccharide constituents, linearity of their backbones, presence of methoxyl groups, uronic acid residues, as well as acidic substituents of neutral monosaccharide units, including pyruvic acid, which contribute to expose negative charges other than sulfate groups [43–46]. Therefore, the different effects on the bacterial biofilm formation could be ascribed to the differences in their structure and also to the presence of positive or negative charges [47]. *C. aerea* extract, mainly composed by negatively charged monomers (glucuronic acid, xylose and iduronic acid), was able to interfere with the initial adhesion of *P. aeruginosa,* as well as the reversible and irreversible adhesion of *S. aureus*. The SP from *A. subulata* was a κ–carregeenans, characterized by one sulfated group for each disaccharide's residue, composed of 1,3α-1,4β-galactans, as previously reported by Chopin et al. [48]. This polysaccharide reduced the cell-wall hydrophobicity of both *P. aeruginosa* and *S. aureus*. Interestingly, the polysaccharide from *A. subulata* was the only extract able to disassemble the preformed biofilm of *P. aeruginosa*. Lectins (such as LecA) are known to play prominent roles in the cellular adhesion and biofilm formation of *P. aeruginosa,* and galactose components of SP from *A. subulata* could act as competitive inhibitors of LecA–ligand [49]. The *H. cornuta* extract consisted of a mixture of λ and ι carrageenans, characterized by a higher content of sulfate groups than polysaccharide of *A. subulata*, which could represent the predominant factor of the antiadhesive activity on *S. aureus*.

The three extracts showed different cytotoxic effects *in vitro* assays, since no cytotoxicity was observed for *H. cornuta* extract, whereas low and high effects were observed for *C. aerea* and *A. subulata* extract, respectively, indicating that κ-carrageenan was more cytotoxic than λ-carrageenan. Conversely, all the extracts did not show any toxicity *in vivo* assays using fish-embryos (*Danio rerio*). These findings are consistent with those reported by Zhou et al., [50], since they observed that λ- and κ-carrageenan exhibited different toxicity *in vitro* or *in vivo* assays.

Our results showed that each algal extract possesses distinctive antiadhesive and antibiofilm properties. The *C. aerea* and *H. cornuta* extract should be used as antiadhesive compounds in several sectors, including food packaging. Since *A. subulata* extract exhibit disassembling properties of *P. aeruginosa* biofilm, it could be used in both the prevention and removal of biofilms.

5. Conclusion

In this study the extracts from *C. aerea*, *A. subulata* and *H. cornuta* were evaluated for their effects on the adhesion and biofilm formation of *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, as model organisms. Without exhibiting any bactericidal effects, the polysaccharides showed dose-dependent inhibitory activity on the biofilm formation of *P. aeruginosa* and *S. aureus*. *C. aerea* extract, a sulfated polysaccharide mainly composed of glucuronic acid, showed the highest antiadhesive activity against *P. aeruginosa,* whereas *H. cornuta* extract, a λ-carrageenan, inhibited the adhesion of *S. aureus*. *A. subulata* extract, a κ-carrageenan, hindered both the adhesion and the development of mature biofilm of *P. aeruginosa*. These extracts could be used to prevent or remove the bacterial biofilms in several medical and non-medical applications.

CRediT authorship contribution statement

Conceptualization, C.Gu. and V.Z.; methodology, S.G., V.Z. and M.G. R.; validation, C.Gu. and S.G.; formal analysis, S.G.; investigation, V.Z., M.G.R., A.S., M.S.N., G.G., M.M., D.S., F.C., C.G., G.C. A.D.M A.M; resources, C.Gu., G.G., M.M., F.C.; data curation C.Gu., V.Z. and A.S.; writing-original draft preparation V.Z., M.G.R. and A.S.; writing-review and editing, C.Gu. and S.G.; supervision, C.Gu.; funding acquisition, C. Gu.

All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102646.

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4.3 Evaluation of the antifouling action of macroalgal extracts on aluminum plates

Manuscript in preparation

In this work the antifouling activity of four extracts deriving from two different algal species, *Ulva* sp. (*Chlorophyta*), and *Asparagopsis* sp. (*Rhodophyta*), applied on aluminum plates tried experimentation at sea, was evaluated. After two months of experiment, the detection of the organisms was carried out using photographs and visual evaluation with the aid of the Fiji Image software. Slabs with different level of deterioration were treated with algal extracts. Preliminary results were promising in limiting the adhesion of marine benthic organisms from an environmental-friendly resource. However, further investigations are needed to verify antifouling mechanism.

4.4 Evaluation of Growth Parameters on *Carpobrotus edulis*, *Kalanchoe daigremontiana* and *Kalanchoe tubiflora* in Relation to Different Seaweed Liquid Fertilizer (SLF) as a Biostimulant

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Evaluation of Growth Parameters on *Carpobrotus edulis, Kalanchoe daigremontiana and Kalanchoe tubiflora* in Relation to Different Seaweed Liquid Fertilizer (SLF) as a Biostimulant

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Keywords

Sustainable agriculture; Biofertilizers; Organic farming; Seaweed extracts; Biocontrol

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A B S T R A C T

The aim of this research was to evaluate the biofertilization capacity of five different species of algae, representative of the three main groups of macroalgae selected from Lake Ganzirri in Messina, Venice Lagoon and Norwegian coasts. The experiments, started in January 2021, were conducted in the greenhouses of CREA-OF in Pescia (Pt), Tuscany, Italy (43°54′N 10°41′E) on *Carpobrotus edulis, Kalanchoe daigremontiana* and *Kalanchoe tubiflora* from seed. The test showed a significant increase in the agronomic parameters analysed in plants treated with algae extracts on succulents cultivated. The test also showed increased control of the pathogen *Pythium debaryanum* in seedlings where the algae extracts were introduced and also an increase in the percentage of seed germination and a reduction in the average germination time. This research work has shown that algal species have great potential for use in the development of biofertilisers in sustainable agriculture in terms of cost-effectiveness, ecological role, possible reduction of synthetic fertilisers and plant protection products, increased soil fertility and microbiological diversity.

Introduction

Marine macroalgae are a source of various biomass use for very different purposes, such as the extraction of bioactive molecules used in pharmaceutical or cosmetics, used as food, feed and agriculture (Leandro *et al.,* 2019; Melo *et al.,* 2020). In the next few years the commercial interest in a product derived from totally sustainable biomass shifts the focus on

some products to those of marine origin, in particular, macroalgae (Biris-Dorhoi *et al.,* 2020); where new products are tested and marketed (Costa *et al.,* 2021), especially in a biorefinery vision and zero waste focus (Zollman *et al.,* 2019).

In particular agriculture products, the majority of which are aimed at increasing the resistance of plants to adverse, in general, abiotic and biotic stress.

Algal extracts are prominent for plant growth and improved the defence from external biological agents for many reasons; the first is the presence in algae of appreciable amounts of trace elements indispensable to plant growth, such as Ca, P, Mg, Fe, Na and K (El-Said and El-Sikaily, 2013; Tuhy *et al.,* 2015); the second reason is the presence of many chemical compounds, naturally present of algae, belong to family of phytohormones (Crouch and Van Staden, 1993;Shoubaky and Salem, 2016); after all, algae have many phycocolloids that substances appreciated for various purposes, of which bio-stimulating activities for plants are also recognized (Kapoore *et al.,* 2021).

Is it important to point out that not all macroalgae have the same set of nutrients, phytohormones and phycocolloids, differentiated not only if look at large groups (brown, green and red algae) but also as regards the differences within the genus (eg *Ulva*) of which not yet the differences are well known (Ali *et al.,* 2021).

Moreover, even within the same species, the levels of nutrients and phytohormones could vary greatly depending on populations, seasonal periods and many other factors, including environmental and biotic factors (Nabil and Cosson, 1996; Khairy and El-Shafay, 2013). Also are important time and type of application, seed germination, foliar direct spray or inside the irrigation water (Vijayakumar *et al.,* 2019). In general, extracts of marine macroalgae are biostimulants for terrestrial plants and raw extracts from seaweed could be better than refined because they restrain many different types of molecules which in refined extracts will be lost (E L Boukhari *et al.,* 2020).

In this study, we aimed to test different seaweed extracts from five different species and test 5 extracts to know which of these was important for the growth of C*arpobrotus edulis* (Figure 1)*, Kalanchoe daigremontiana, Kalanchoe tubiflora.*

The seaweed extracts used in the present study were prepared from five different species, representative of the three major macroalgal groups. For the Phaeophyceae (Brown algae) *Saccharina latissima* (A) and *Sargassum muticum* (C) were chosen, about Chlorophyta (Green algae) was chosen *Ulva ohnoi* (B) and for Rhodophyta (Red algae) *Hypnea cornuta* (D) and *Agardhiella subulata* (E). All these macroalgae were used because of utilization of unwanted biomass, alien or invasive species and, in the case of *S. latissima*, production waste for human consumption.

Materials and Methods

The experiments, started in January 2021, were conducted in the greenhouses of CREA-OF in Pescia (Pt), Tuscany, Italy (43°54′N 10°41′E) on *Carpobrotus edulis, kalanchoe daigremontiana* and *kalanchoe tubiflora* from seed. The seeds were placed in a 54 hole plateau, in potting soil for sowing (blond peat 40%, black peat 60%, silica sand 80 l/m^3, fertilization 500/m^3 NPK, pH 5.7); 60 seeds per thesis, divided into 6 replicas of 10 seeds each. The plants were watered 2 times per day, 7 days a week and grown for 6 months. The plants were irrigated with drip irrigation. The irrigation was activated by a timer whose

program was adjusted weekly according to climatic conditions and the fraction of leaching. On June 15, 2021, seeds germination, average germination time, plants height, leaves number, vegetative and radical weight, were recorded. In addition, plant infection by *Pythium debaryanum* selected from *Solanum lycopersicum* seedlings and inoculated on succulents plants was evaluated in the experiment.

The algae were collected from different places all around Europe and for different purposes that have been mentioned above. *Saccharina latissima* from crop waste in Norway (58°17'33.3"N 6°38'58.3"E), after sampling, was quickly dring; *Sargassum muticum* from Venice lagoon (45°25'42.6" N 12°19'50.7"E) (Italy); *Ulva ohnoi*, *Hypnea cornuta* and *Agardhiella subulata* from Ganzirri lake (38°15'28" N 15°36'37" E) (Italy).

After sampling algae were brought to the laboratory and rapidly washed with fresh water to remove epiphytes and sand. The fresh alga was dried in the laboratory stove at 40°C for 48h and kept dried until extraction, preserving dryness through silica gel.

Dried macroalgae were prepared for extraction with the modified protocol from Rama, about the preparation of Liquid Seaweed Fertilizer (Rama 1990).

Five different solutions were prepared, each solution was prepared with 500 ml of distilled water and 25 g of dried seaweed (ratio 1:20 DW/V) at 80°C for 3h. The residue biomass was removed by the use of cotton cloth and the liquid solution gives, as a result, a 100% SLF (Seaweed Liquid Fertilizer), used with a different concentration on *Carpobrotus edulis*, *Kalanchoe daigremontiana, Kalanchoe tubiflora*. To preserve the fertilizer was add 1g/l of citric acid and keep at 4°C until dilution and use.

Results and Discussion

The test showed a significant increase in the agronomic parameters analysed in plants treated with algae extracts on *Carpobrotus edulis*, *Kalanchoe daigremontiana* and *Kalanchoe tubiflora*. The test also showed increased control of the pathogen *Pythium debaryanum* in seedlings where the algae extracts were introduced. There was also an increase in the percentage of seed germination and a reduction in the average germination time in the theses treated with algae. In particular, the trials showed a more significant effect on vegetative and root development in the theses treated with *Hypnea cornuta* and *Agardhiella subulata* on all three succulent species in cultivation. All plants treated with the algae extracts showed a significant increase in the height and number of leaves per plant, the vegetative and root weight of the plants, a reduction in attack by the pathogen *Pythium debaryanum*, an increase in seed germination and a reduction in the average germination time.

Specifically, in (Table 1), it is shown that on *Carpobrotus edulis*, treatment (E) significantly improved the plant height with 14.35 cm, compared to 13.81 cm (D), 11.17 cm (C), 10.49 cm (B), 9.61 cm (A) and 7.92 cm of the untreated control. The use of algae extracts also significantly increased the number of leaves per plant with 6.80 (E), 6.20 (D), 5.40 (A), 5.00 (B), 4.80 (C) and 4.60 of (CTRL). It also increased the vegetative weight, 27.71 g (E), 26.94 g (D), 24.89 g (B), 24.48 g (A), 24.27 (c) and 22.94 of the untreated control and root weight 20.27g (E), 19.93 g (D), 18.43 g (C), 18.33 g (B), 17.59 g (A) and 16.83 g (CTRL) (Figure 2). The trial also showed that treatments based on algae extracts can have a biocontrol effect on the *Pythium dabaryanum* pathogen. Indeed, in theses sprayed with algae extracts, 1.00 plant was affected by Pythium in (E), 1.20 in (D),

1.40 in (A) and (C), 2.20 in (B), 3.40 (CTRL). In relation to seed germination, theses (E) and (D) were the best with 8.20 and 7.80 seeds germinated, followed by (B) and (C) with 6.40, (A) with 5.60 and (CTRL) with 4.40. Regarding the mean germination time, (E) and (D) were the best theses with 18.60 and 18.80 days, (C) 21.80 days, (A) 21.40 days, (B) 21.00 days and the control with 24.20 (CTRL).

In (Table 2) on *Kalanchoe daigremontiana*, it is noted that treatments (E) and (D) significantly improved the plant height with 8.86 cm and 8.67 cm respectively, followed by (B) with 7.93 cm, (A) with 7.91 cm and (C) with 7.71 cm. The control was the treatment with the lowest plant height with 7.04 cm. Regarding the number of leaves, treatments (E) and (D) were the best with 8.60 and 8.00 leaves, followed by (C) with 6.40, (A) and (B) with 6.00 and the untreated control with 5.20. The application of algae extracts also resulted in an increase in vegetative weight (Figure 3), 18.83 g (E), 17.57 g (D), 16.09 g (C),15.48 g (A), 15.04 (CTRL) and 14.92 (b) and root weight 14.55 g (E), 13.85 g (D), 12.53 g (C), 12.19 g (D), 11.45 g (A) and 10.05 g (CTRL) (Figure 4). Also on *K. daigremontiana*, it was evident that the algae extracts showed a biocontrol effect on Pythium. In particular, in all the theses treated with the extracts there was a significant reduction in the pathogen's attack compared to the control. In terms of seed germination, thesis (D) was the best with 9.00 germinated seeds, followed by (E) with 8.80, (B) and (C) with 8.40, (A) with 8.00 and (CTRL) with 7.40. Regarding the mean germination time, (E) and (D) were the best theses with 15.30 and 16.40 days, (A) and (C) 17.20 days, (B) 17.60 days and the control with 18.00 (CTRL).

On *Kalanchoe tubiflora* in (Table 3), it can be seen that treatment (E) was the best for plant height with 8.87 cm, succeeded by (D) with 8.32 cm and (A), (C) and (B) with 7.73 cm, 7.64 cm and 7.58 cm respectively.

Finally, the untreated control with 7.05 cm. For the number of leaves, treatment (D) was the best with 8.60, then (E) with 8.40, (B) with 7.80, (A) and (C) with 7.40. The last one was the untreated control with 7.20. An increase in vegetative weight was also shown on *K. tubiflora* particularly in treatment (E) with 16.43 g, which was followed by (D) with 16.17 g, (C), (B) and (A) with 15.60 g, 15.42 g and 15.39 g respectively, lastly the control with 14.70 g. Treatment (E) was also the best for root weight with 14.68 g, closely followed by (D) with 14. 01 g, (C) and (B) with 13.55 g and 13.52 g respectively, (A) with 12.64 g and (CTRL) with 11.26 g. No treatment showed any significant effect on the control of *Pythium debaryanum*, while with regard to seed germination treatments (E) and (D) with 9.20 and 8.80 were the best, compared to the other plant extracts and the control. Treatment (E) was also the best in terms of average germination time with 13.20 days.

In modern years, there has been a tendency to increase efficiency in the use of synthetic fertilisers and a move back to the use of plant algae products to enhance plant quality and the ability to utilise plant nutrients (Thajuddin and Subramanian, 2005). Since the 1950s, the use of algae has been superseded by the use of commercial extracts that provide useful molecules for plants. The efficiency of algae as bio-stimulants is dependent on the content and composition of molecules that can improve plant metabolism under stressful conditions (Saadatnia and Riahi, 2009).

Some of the hormones most commonly found in algae extracts include cytokinins, auxins, gibberellins and abscisic acid, as well as other hormone-like substances (Song *et al.,* 2005).

The effects found in the use of algae in plant cultivation include: (i) increased porosity and production of adhesive substances; (ii)

excretion of substances that stimulate the production of phytohormones (auxin, gibberellin); (iii) increased water retention due to the gelatinous structure of algae; (iv) increased soil biomass as a result of the death and desiccation of algal structures; (v) reduced soil salinity; (vi) reduced weed development; and (vii) increased phosphate and organic acid content in the soil (Sahu *et al.,* 2012; Rodriguez *et al.,* 2006; Wilson, 2006). Beneficial effects of algae inoculation in plants have been found on barley, oats, tomato, radish, cotton, sugarcane, maize, chilli and lettuce and also on ornamentals such as cacti and succulents (Saadatnia and Riahi, 2009; Liesack *et al.,* 2000; Nayak *et al.,* 2001; Prisa, 2019; Prisa, 2020).

Table.1 Evaluation of liquid seaweed fertilizer on agronomic and pathological characters of *Carpobrotus edulis*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	PI (n°)	SG (n°)	GT (days)
CTRL	7,92 f	4,60 c	22,94 d	16,83 e	3,40 a	4,40 d	24,20 a
A	9,61 e	5,40 b	24,48 c	17,59 d	1,40 c	5,60 c	21,40 b
B	10,49 d	5,00 bc	24,89 c	18,33 c	2,20 b	6,40 b	21,00 b
C	11,17 c	4,80 bc	24,27 c	18,43 c	1,40 c	6,40 b	21,80 b
D	13,81 b	6,20 a	26,94 b	19,93 b	1,20 c	7,80 a	18,80 c
E	14,35 a	6,80 a	27,71 a	20,27 a	1,00 c	8,20 a	18,60 c
ANOVA	***	***	***	***	***	***	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL): control; (A): *Saccharina latissima;* (B): *Ulva ohnoi*; (C): *Sargassum muticum*; (D): *Hypnea cornuta*; (E): *Agardhiella subulata*; PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; PI: plants infected by *Pythium debaryanum;* SG: number of seeds germinated; GT: germination time.

Table.2 Evaluation of liquid seaweed fertilizer on agronomic and pathological characters of *Kalanchoe daigremontiana*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	PI (n°)	SG (n°)	GT (days)
CTRL	7,04 c	5,20 c	15,04 de	10,05 f	1,60 a	7,40 d	18,00 a
A	7,91 b	6,00 bc	15,48 d	11,45 e	0,40 b	8,00 c	17,20 a
B	7,93 b	6,00 bc	14,92 e	12,19 d	0,00 b	8,40 bc	17,60 a
C	7,71 b	6,40 b	16,09 c	12,53 c	0,40 b	8,40 bc	17,20 a
D	8,67 a	8,00 a	17,57 b	13,85 b	0,00 b	9,00 a	16,40 b
E	8,86 a	8,60 a	18,83 a	14,55 a	0,20 b	8,80 ab	15,30 b
ANOVA	***	***	***	***	***	***	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL): control; (A): *Saccharina latissima;* (B): *Ulva ohnoi*; (C): *Sargassum muticum*; (D): *Hypnea cornuta*; (E): *Agardhiella subulata*; PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; PI: plants infected by *Pythium debaryanum;* SG: number of seeds germinated; GT: germination time.

Table.3 Evaluation of liquid seaweed fertilizer on agronomic and pathological characters of *Kalanchoe tubiflora*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	PI (n°)	SG (n°)	GT (days)
CTRL	7,05 d	7,20 c	14,70 d	11,26 e	0,20 a	7,20 b	15,60 a
A	7,73 c	7,40 c	15,39 c	12,64 d	0,00 a	7,60 b	14,80 b
B	7,58 c	7,80 bc	15,42 c	13,52 c	0,00 a	7,20 b	14,20 bc
C	7,64 c	7,40 c	15,60 c	13,55 c	0,20 a	7,60 b	14,00 cd
D	8,32 b	8,60 a	16,17 b	14,01 b	0,20 a	8,80 a	13,40 de
E	8,87 a	8,40 ab	16,43 a	14,68 a	0,20 a	9,20 a	13,20 e
ANOVA	***	***	***	***	ns	***	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL): control; (A): *Saccharina latissima;* (B): *Ulva ohnoi*; (C): *Sargassum muticum*; (D): *Hypnea cornuta*; (E): *Agardhiella subulata*; PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; PI: plants infected by *Pythium debaryanum;* SG: number of seeds germinated; GT: germination time.

Fig.1 Detail of germination plateaus and germinated *Carpobrotus edulis* seedlings in nurseries at CREA-OF in Pescia

Fig.2 Effect on vegetative and roots biomass of *Carpobrotus edulis* by *Hypnea cornuta* (D) and *Agardhiella subulata* (E) compared to the untreated control (CTRL)

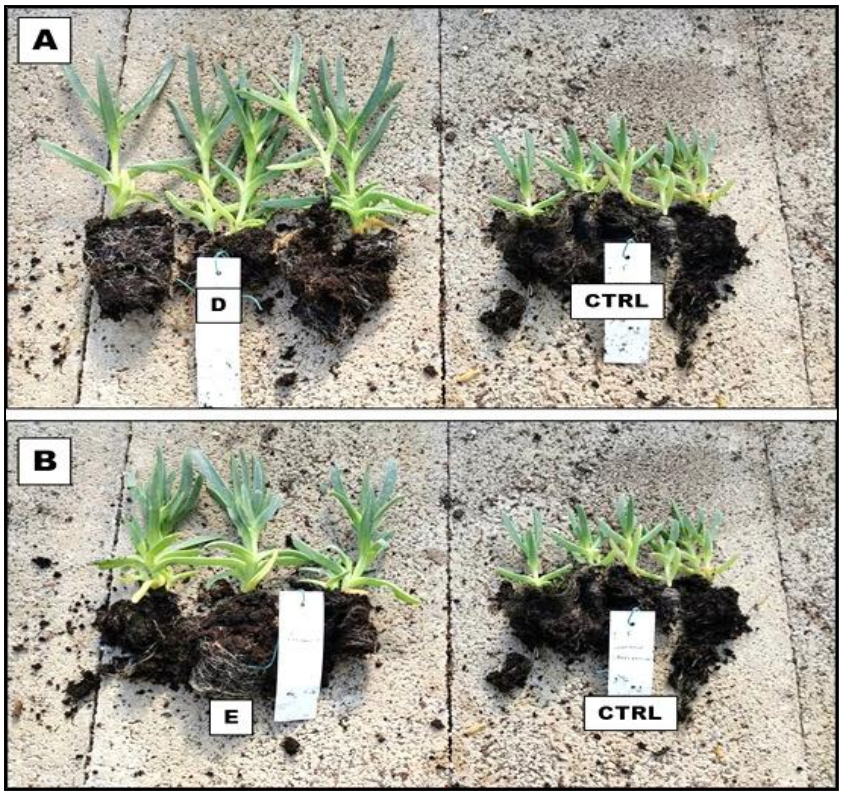


Fig.3 Effect on vegetative and roots biomass of *kalanchoe tubiflora* by *Hypnea cornuta* (D) and *Agardhiella subulata* (E) compared to the untreated control (CTRL)

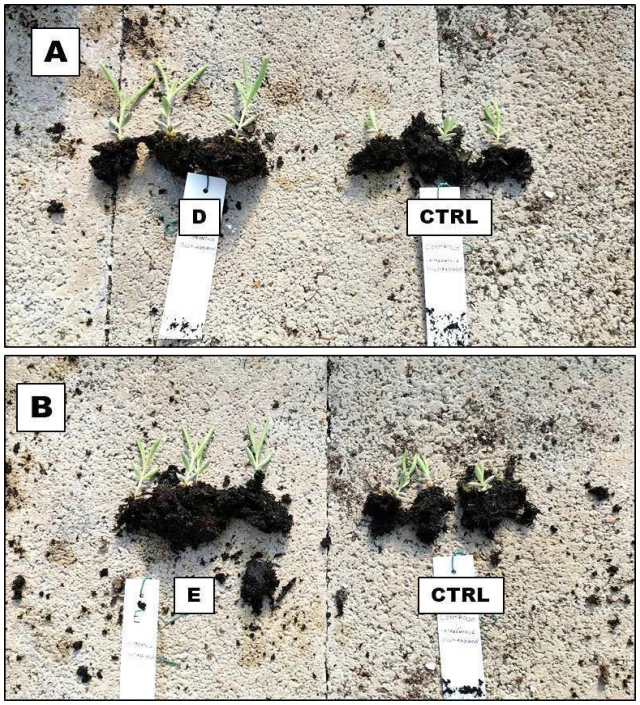
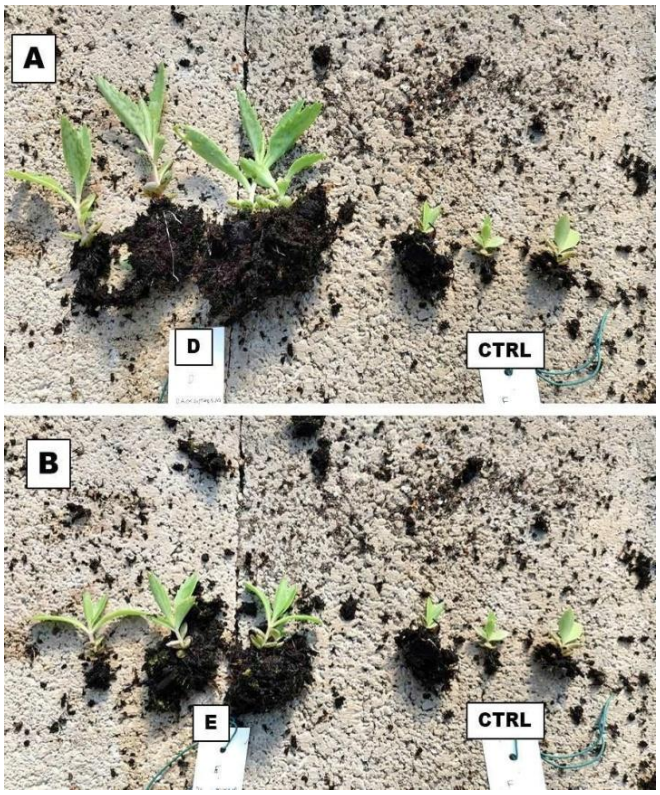


Fig.4 Effect on vegetative and roots biomass of *Kalanchoe daigremontiana* by *Hypnea cornuta* (D) and *Agardhiella subulata* (E) compared to the untreated control (CTRL)



In this experiment on *Carpobrotus edulis*, *Kalanchoe daigremontiana* and *Kalanchoe tubiflora*, the ability of selected algae extracts from Lake Ganzirri (ME), Venice Lagoon and Norwagian coasts, to significantly improve and increase plant quality, increase vegetative and root biomass, control pathogens such as *Pythium debaryanum* and improve seed germination is confirmed. Among all five algal extracts, two red algae present in Lake Ganzirri, proved effective in all measurements: *Hypnea cornuta* but, most of all, *Agardhiella subulata*.

Effects that can certainly be related to an increase in nutritional status due to increased nutrient uptake and the structural capabilities of the algae to provide greater water retention. The increased control of seedling pathogens can certainly be related to the structural and metabolic component of the algae that inhibits the development of fungi and bacteria (Rao and Burns, 1991).

Another aspect that should not be underestimated could be the possible modification of the microbial balance in the soil following the inoculation of blue-green algae or other types of algae (Ibrahim *et al.,* 1971; Acea *et al.,* 2001). Some studies have shown that algae inoculation can lead to an increase of up to eight times in microbial communities. The results suggest a carbon and energy surplus due to algae polysaccharides as one of the reasons behind the increase in heterotrophic microbial populations (Rogers and Burns, 1994; Anderson and Gray, 1991).

This research work has shown that algal species have great potential for use in the development of biofertilizers in sustainable agriculture in terms of cost-effectiveness,

ecological role, possible reduction of synthetic fertilisers and plant protection products, increased soil fertility and microbiological diversity.

In particular, this trial is of particular interest for the selection of new algal products that can be used as plant biofertilizers to improve growth and reduce biotic and abiotic stresses in plants. in particular, were tested five different extracts and, as not expected result, *Agardhiella subulata* extract was the most effective biostimulant for all three plant tested at the expense of brown algae, with normally greater biostimulating capacities and green algae that produce flourishing biomass.

Further experiments will be carried out to evaluate the selection capabilities of new algal extracts detected in the Ganzirri Lake of Messina (Italy) in order to better understand the biostimulation potential and the possible reduction of water and salt stresses of plants grown in pots.

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4.5 Evaluation of the bio-stimulating activity of lake algae extracts on edible cacti *Mammillaria prolifera* and *Mammillaria glassii*

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Evaluation of the Bio-Stimulating Activity of Lake Algae Extracts on Edible Cacti *Mammillaria prolifera* and *Mammillaria glassii*

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Abstract: The research aimed to test different seaweed extracts derived from three macroalgae representatives, namely, *Rhodophyta, Chlorophyta* and *Phaeophyceae*, as a bio-fertiliser for the growth of *Mammillaria prolifera* and *Mammillaria glassii* and the production of edible fruits. The experiments started in September 2021 and were conducted in the greenhouses of CREA-OF in Pescia (PT). Three different algae, namely, *Hypnea cornuta* (*Rhodophyta*), *Ulva ohnoi* (*Chlorophyta*), collected from the brackish lake Ganzirri, in Messina, and *Sargassum muticum* (*Phaeophyceae*) from Venice lagoon, were tested. The experimental trial showed a significant improvement in the agronomic parameters analysed for the growth and production of cactus plants and fruits treated with the selected algae. A significant increase was found in the sugar, vitamin A, vitamin C and vitamin E content of the fruits of treated plants. In particular, the thesis with *Ulva ohnoi* was the best for plant growth and fruit production with a higher sugar and vitamin content. This experiment confirms the algae's ability to stimulate soil microflora and microfauna, promoting nutrient uptake, participating in organic matter mineralisation processes and significantly influencing the nutraceutical compounds in the fruits.

Keywords: sustainable agriculture; bio-fertilisers; organic farming; seaweed extracts; edible cactus; ornamental plants

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1. Introduction

Anthropic processes act as one of the prominent effects accentuated by climate change. Consequently, drought events are increasing, causing a relevant loss in crop yield and water availability for cultivation [1,2]. Considering this, improving resistance ability and agricultural production is vital, and is a challenging task, but is needed to meet the growing food demand due to population growth [3]. Optimal mineral nutrition increases plant resilience to different stresses and enhances products' quality. Nevertheless, chemical fertilisation causes high economic and ecological costs [4]. Fertilisers from macroalgae products that respond to the requirements for agriculture could be used in biological agriculture as they are not synthetic, and at the same time, they can provide greater yields and mitigate the effects of climate change, stimulating plant nutrition processes [5–7]. Additionally, macroalgae fertiliser provides a wide range of plant growth regulators, thus turning this fertiliser into a more noble biostimulant [8–10]. Therefore, adopting macroalgae as a bio-fertiliser is a good solution for sustainable agriculture because it combines an economical source to obtain fertiliser with the removal of some macroalgae biomass, which may occur especially in eutrophic environments [11,12]. Macroalgae can produce immense biomass in nutrient-rich environments, such as estuaries, natural and artificial lakes, and, more generally, in transitional environments that often have a substantial anthropogenic impact, which is often dredged as a waste so as not to affect human activities. Previous experiments using algae on plants such as maize and rice resulted in increased growth in terms of dry

matter and the C and N content of the soil [13–15]. In addition, an increase in shoot height in the treated maize theses and an increase in root and shoot development in rice were also observed. Tests on *Amaranthus* spp. showed an increase in plant height and biomass produced in the theses treated with algae [16,17]. Further trials on *Portulaca grandiflora*, *Aloe Barbadensis Miller* and *Lobivia* spp. showed a significant effect on increased vegetative and root development of the plants and on the mineral and sugar content in stems and leaves [18–20]. Algae extracts have been used for a long time because they can improve plant growth and increase resistance to various biotic and abiotic stresses. In particular, algae contain considerable amounts of indispensable mineral elements such as calcium, phosphorus, iron and potassium; various compounds with hormonal and stimulating activity are also present in algae [21,22]. In addition, algae contain colloid substances used for various purposes, e.g., for their proven stimulating activity [23,24]. Agriculture consumes a considerable amount of nutrients, especially nitrogen and phosphorus, to meet the food demands of an actively growing global population. However, nutrients to sustain the agricultural system are supplied by unsustainable processes, and most fertilisers introduced into agriculture are dispersed into the surrounding ecosystem, which, when dispersed into the soil, bind with the organic matter complexly, making plants unable to assimilate them, thus burdening the environmental impact of the agricultural system [25]. Therefore, developing sustainable alternatives for nutrient production to sustain the current agricultural production chain is a priority. Among the alternatives, the high content of micro- and macronutrients makes microalgae biomass a promising source of bio-fertiliser. From different algal species, bio-fertilisers with stimulating properties are generally produced individually or in combination with bacteria and mycorrhizae. Microalgae and cyanobacteria products are generally developed by means of photobioreactors, such as *Arthrospira* spp., *Dunaliella* spp., *Anabaena* spp., *Phaeodactylum* spp., *Pleurochrysis* spp., *Chlorella* spp. and *Nannochloropsis* [26–28]. These products, once obtained, can be applied to plants in fertigation or combined with fertilisers in integrated agriculture protoclimates. Stimulant products based on *Ascophyllum nodosum* are used today as stimulants for increasing plant protection from biotic and abiotic stresses in ornamentals [29]. Various microalgae species have been studied for their application as bio-fertilisers, with soil-stabilising effects, increased nutrient content, and increased water retention capacity. However, the mechanism responsible for bio-fertilisation has not yet been fully elucidated [30]. Microalgae have a high nutrient content, which must be accessible to plants, so the microbiome of the rhizosphere degrade the biomass of the microalgae to release constituent nutrients or undergo natural degradation to allow a sustained release of nutrients. Alternatively, microalgae biomass could actively interact with plants and, in the case of nitrogen-fixing cyanobacteria, induce the release of bioavailable forms of nitrogen in exchange for carbon compounds from the plant [31]. In the latter case, the microalgae would also have to interact with the rhizosphere microbiome actively, and, therefore, compatibility and survival are not guaranteed. Combined with the possibility of providing energy resources, microalgae biomass has a broader effect on plant growth by synthesising psychostimulant molecules such as hormones [32,33]. Phytohormones found in extracts of microalgae biomass include auxins, gibberellin-like molecules and abscisic acid, affecting the growth and plant development due to stimulatory activity on various metabolic processes such as photosynthesis, respiration, nucleic acid synthesis and nutrient assimilation [34].

The research aimed to test different seaweed extracts, derived from three macroalgae representatives of the *Rhodophyta*, *Chlorophyta* and *Phaeophyceae*, as a bio-fertiliser for the growth of *Mammillaria prolifera* and *Mammillaria glassii* and the production of edible fruits, and to evaluate the interactions between algae and the soil microbiome (Figure 1).

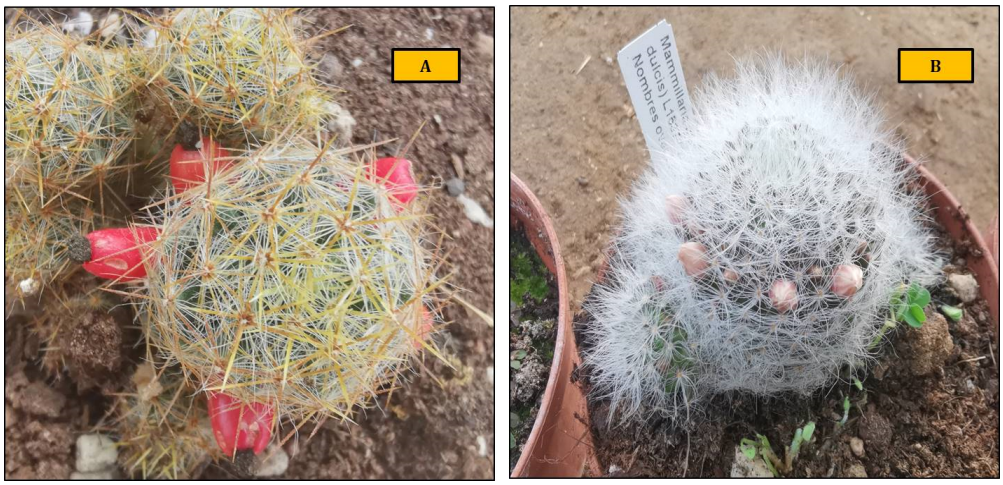

Figure 1. A view of *Mammillaria prolifera* (**A**) and *Mammillaria glassii* (**B**) plants in CREA-OF greenhouses.

2. Results

The experimental trial at the CREA-OF greenhouses in Pescia showed a significant improvement in the agronomic parameters analysed on the growth and production of *Mammillaria prolifera* and *Mammillaria glassii* fruits treated with selected algae from Lake Ganzirri (ME) and the Venice Lagoon. Treatments with selected algae from these lakes resulted in an increase in the vegetative and root development of the plants, with an increase in height, circumference and number of spines and flowers in the epigeal part and root length in the hypogeal part of these plants. Significant effects were also evident in the increase in the microbial biomass of the algae-treated theses. Finally, a significant increase in sugar, vitamin A, vitamin C and vitamin E content was found in the fruits of the plants treated with the stimulating algae. In particular, the thesis with *Ulva ohnoi* was the best of all, both in terms of plant growth and the sugar and vitamin content of the fruits. The results show the differences in plant growth, fruit production and sugar and vitamin content obtained with the treatments of (AG) Ecklonia maxima, (HC) Hypnea cornuta, (UO) Ulva ohnoi and (SM) Sargassum muticum compared with the fertilised control (CTRL).

Mammillaria prolifera plant height was significantly increased by UO at 9.38 cm, SM at 8.25 cm, AG at 8.23 cm, HC at 8.14 cm and finally (control) at 7.46 cm (as shown in Table 1). With 4.4 new suckers, UO was the best thesis, followed by SM with 3.4, HC with 3.2, AG with 2.6 and CTRL with 1.6. Significant development of the vegetative part can be seen in UO, followed by AG and HC at 34.97 and 34.86 g, respectively, SM at 34.37 g, and finally, CTRL at 32.89 g (Figure 2). Figure 3 shows the same trend in root weight, with the UO root weight being 25.75 g, the SM and the HC root weights being 23.14 g and the AG root weight being 22.93 g. In terms of plant circumference, UO was also the best thesis with 6.63 cm, followed by SM at 5.86 cm, HC at 5.80 cm, AG at 5.29 m and CTRL at 4.86 cm. Ulva ohnoi was also the best thesis for flower production, 22.6, compared to 15.4 (AG), 14.8 (HC), 14.4 (S, M) and 13.6 (CTRL). In flower duration, UO was the best thesis with 4.6 days, followed by AG and HC with 3.6 days, (M with 3.4 days, and finally, the control with 2.4 days. No major differences in the development of the spines are evident between the various experimental theses.

Table 1. Evaluation of liquid seaweed fertiliser on agronomic characters of *Mammillaria prolifera*.

Groups	PH (cm)	SN (n°)	VW (g)	RW (g)	PC (cm)	FN (n°)	FL (days)	NT (n°)	LT (mm)
CTRL	7.46 c	1.6 d	32.89 d	21.55 c	4.86 d	13.6 c	2.6 c	95.0 a	2.58 a
AG	8.23 b	2.6 c	34.97 b	22.93 b	5.29 c	15.4 b	3.6 b	96.4 a	3.28 a
HC	8.14 b	3.2 bc	34.86 b	23.14 b	5.80 b	14.8 b	3.6 b	95.6 a	3.36 a
UO	9.38 a	4.4 a	37.72 a	25.75 a	6.63 a	22.6 a	4.6 a	95.0 a	10.3 a
SM	8.25 b	3.4 b	34.37 c	23.14 b	5.86 b	14.4 bc	3.4 b	96.0 a	2.74 a
ANOVA	***	***	***	***	***	***	***	*ns*	*ns*

Significance according to Tukey's analysis (HSD) multiple-range test ($p = 0.05$). Legend: (CTRL): control; (AG): *Ecklonia maxima*; (HC): *Hypnea cornuta*; (UO): *Ulva ohnoi*; (SM): *Sargassum muticum*; PH: plant height; SN: suckers number; VW: vegetative weight; RW: roots weight; PC: plants circumference; FN: flowers number; FL: flowers life; NT: Thorns number; LT: thorns length. Different letters in the same column mean significant differences between varieties.

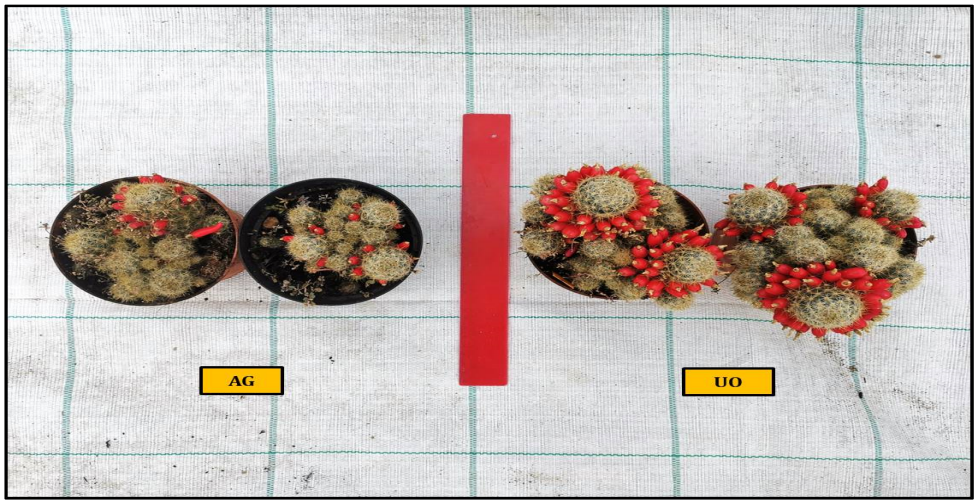

Figure 2. Comparison of vegetative growth and fruit production of *Ulva ohnoi* (UO) and *Ecklonia maxima* (AG) in *Mammillaria prolifera*.

Figure 3. Comparison of root growth in *Mammillaria prolifera* plants treated with *Ulva ohnoi* (UO) and *Ecklonia maxima* (AG).

There were no significant changes in the pH of the substrate (Table 2) of Mammillaria prolifera. As a result of the test, algae-treated substrates demonstrated a higher microbial

presence with 1.29×10^3 cfu/g, followed by HC with 1.27×10^3 cfu/g, SM with 1.26×10^3 cfu/g, AG with 1.23×10^3 cfu/g and finally the control with 1.25×10^2 cfu/g. The thesis UO had the highest fruit production with 14, compared to HC with 7.8, AG and SM with 7.4 and the control with 5.8. There was also a similar trend in fruit weight, with (UO) having 3.72 g (the best fruit weight), HC having 3.33 g and SM having 3.29 g, respectively, whereas AG having 3.17 g and CTRL having 2.50 g. In terms of sugar content, UO and SM topped the list with 4.85 g and 4.82 g, respectively, followed by HC with 4.76 g, AG with 4.68 g and CTRL with 4.57 g. Vitamin A content in the SM thesis was significantly lower than in the others, while vitamin C and vitamin E contents in the UO and SM thesis were the highest.

Table 2. Evaluation of microbial biomass, production, sugar and vitamin content in *Mammillaria prolifera fruits*.

Groups	PH	MC (cfu/g)	FN (n°)	FW (g)	SC (g)	Vit. A (mg)	Vit. C (mg)	Vit. E (mg)
CTRL	6.48 a	1.25×10^2 d	5.8 c	2.50 d	4.57 d	7.36 a	8.45 c	0.024 c
AG	6.48 a	1.23×10^3 c	7.4 b	3.17 c	4.68 c	7.24 a	8.62 b	0.026 c
HC	6.48 a	1.27×10^3 b	7.8 b	3.33 b	4.76 b	7.26 a	8.72 b	0.054 ab
UO	6.44 a	1.29×10^3 a	14.0 a	3.72 a	4.85 a	7.32 a	8.95 a	0.064 a
SM	6.44 a	1.26×10^3 b	7.4 b	3.29 b	4.82 a	6.98 b	8.86 a	0.044 b
ANOVA	*ns*	***	***	***	***	***	***	***

Significance according to Tukey's analysis (HSD) multiple-range test ($p = 0.05$). Legend: (CTRL): control; (AG): *Ecklonia maxima*; (HC): *Hypnea cornuta*; (UO): *Ulva ohnoi*; (SM): *Sargassum muticum*; PH: acidity or basicity of the substrate; MC: microbial count; FN: fruit number; FW: fruit weight; SC: sugar content. Different letters in the same column mean significant differences between varieties.

The plant height of UO of Mammillaria glassii was significantly increased at 6.48 cm (Table 3), followed by SM at 6.41 cm, HC at 6.31 cm, AG at 6.29 cm and finally, CTRL at 5.38 cm. There were 3.4 new suckers in UO, AG and HC, followed by SM with 1.6 and the control with 1.2. As shown in Figure 4, UO had a better weight for the vegetative part at 29.23 g, followed by HC at 28.27 g, AG at 27.87 g, SM at 27.48 g and CTRL at 26.46 g. Root weight follows the same trend with UO weighing 20.13 g, AG and HC weighing 18.87 g and 18.86 g, respectively, and, finally, SM depicting 17.74 g, as well as the untreated control of 17.68 g. Plant circumference was also highest for UO at 6.25 cm, followed by HC and SM at 5.94 cm and 5.83 cm, respectively, AG at 5.27 cm and CTRL at 4.63 cm. In Mammillaria glassii, Ulva ohnoi had the highest flower production, 15.0, compared with 12.8 for AG and HC, 12.4 for SM and 8.6 for CTRL. The flower duration in UO was highest at 4.4 days, followed by AG and HC at 3.8 days, 3.6 days and, finally, 2.8 days for SM and CTRL. Differences in the growth of thorns were evident, with UO showing the best results having 124.2 spines of 3.06 cm in length, respectively.

Table 3. Evaluation of liquid seaweed fertiliser on agronomic characters of *Mammillaria glassii*.

Groups	PH (cm)	SN (n°)	VW (g)	RW (g)	PC (cm)	FN (n°)	FL (days)	NT (n°)	LT (mm)
CTRL	5.38 d	1.2 c	26.46 e	17.68 c	4.63 d	8.6 c	2.8 c	97.0 c	1.44 d
AG	6.29 c	2.2 b	27.87 c	18.87 b	5.27 c	12.8 b	3.8 ab	111.8 b	2.22 c
HC	6.31 c	2.2 b	28.27 b	18.86 b	5.94 b	12.8 b	3.8 ab	100.4 c	2.42 b
UO	6.48 a	3.4 a	29.23 a	20.13 a	6.25 a	15.0 a	4.4 a	124.2 a	3.06 a
SM	6.41 b	1.6 bc	27.48 d	17.74 c	5.83 b	12.4 b	3.6 b	99.0 c	2.16 c
ANOVA	***	***	***	***	***	***	**	***	***

Significance according to Tukey's analysis (HSD) multiple-range test ($p = 0.05$). Legend: (CTRL): control; (AG): *Ecklonia maxima*; (HC): *Hypnea cornuta*; (UO): *Ulva ohnoi*; (SM): *Sargassum muticum*; PH: plant height; SN: suckers number; VW: vegetative weight; RW: roots weight; PC: plants circumference; FN: flowers number; FL: flowers life; NT: Thorns number; LT: Thorns length. Different letters in the same column mean significant differences between varieties.

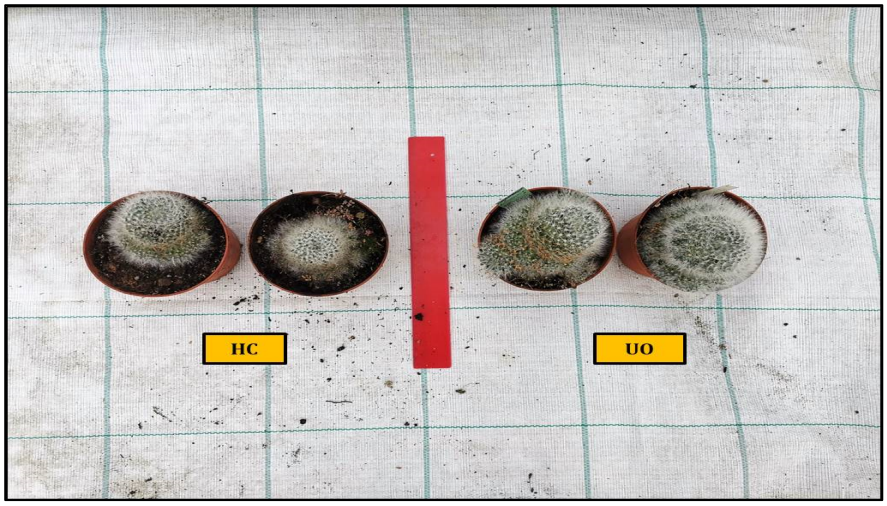

Figure 4. Comparison of the vegetative growth of *Ulva ohnoi* (UO) and *Hypnea cornuta* (HC) in *Mammillaria glassii*.

Mammillaria glassii substrate pH hovered around 6.4 (Table 4), but no significant difference was observed. One of the most interesting aspects of this test was the microbial colonisation of the algae-treated substrates; in particular, UO was the thesis with the highest microbial presence of 1.64×10^3 cfu/g, followed by HC and SM with 1.32×10^3, AG with 1.26×10^3 and CTRL with 1.25×10^2. As compared to all of the other experimental theses, UO produced the best fruit, with 11.4. According to fruit weight, UO produced the best weight at 4.07 g, followed by SM at 3.84 g, AG at 3.72 g, HC at 3.29 g and CTRL at 2.43 g. With 4.55 g of sugar and 7.30 mg of vitamin, UO had the highest levels of sugar and vitamin content. Vitamin C content in all theses with stimulating algae was higher than that in control, while vitamin E content in UO was the highest (0.06 mg).

Table 4. Evaluation of microbial biomass, production, sugar, and vitamin content in *Mammillaria glassii* fruits.

Groups	PH	MC (cfu/g)	FN (n°)	FW (g)	SC (g)	Vit. A (mg)	Vit. C (mg)	Vit. E (mg)
CTRL	6.46 a	1.25×10^2 d	5.6 c	2.43 e	3.70 e	6.14 d	7.36 b	0.03 bc
AG	6.42 a	1.26×10^3 c	8.2 b	3.72 c	4.26 c	7.11 b	7.90 a	0.02 c
HC	6.44 a	1.32×10^3 b	7.6 b	3.29 d	4.44 b	7.13 b	7.93 a	0.03 bc
UO	6.46 a	1.64×10^3 a	11.4 a	4.07 a	4.55 a	7.30 a	8.05 a	0.06 a
SM	6.48 a	1.32×10^3 b	7.4 b	3.84 b	4.18 d	6.98 c	7.95 a	0.04 b
ANOVA	*ns*	***	***	***	***	***	***	***

Significance according to Tukey's analysis (HSD) multiple-range test ($p = 0.05$). Legend: (CTRL): control; (AG): *Ecklonia maxima*; (HC): *Hypnea cornuta*; (UO): *Ulva ohnoi*; (SM): *Sargassum muticum*; PH: acidity or basicity of the substrate; MC: microbial count; FN: fruit number; FW: fruit weight; SC: sugar content. Different letters in the same column mean significant differences between varieties.

3. Discussion

It seems that the term biostimulant was coined to describe those substances that promote plant growth without being nutrients, soil conditioners or pesticides. Interestingly, the first discussion of biogenic stimulants is attributed to a Russian named Filatov and dates back to the 1930s [35,36]. This definition refers to specific biological material derived from various organisms, including plants, which have been exposed to stressors and could influence metabolic and energetic processes in humans, animals and plants [37–39]. Depending on the geographical region and species, climate change can have positive and negative effects on crops and can impact plant growth, fruit development, flower intensity and structure. Despite being cultivated and well-watered, the impact on plants is clearly

evident and leads them to become smaller, shorter and less drought-resistant. In addition, the amount of CO_2 absorbed through photosynthesis is reduced, resulting in a significant reduction in plant productivity. Climate change can also lead to the development of serious plant diseases caused by fungi and insects that usually live in different areas [40]. Numerous studies have been conducted to identify the functional molecules contained in algae extracts. According to Prisa [41] and Mulberry [42], the use of algae can stimulate plant growth by influencing tissue mineral content, flower production and plant quality. Among the bioactive substances that seem to be responsible for this growth, hormones are certainly the most likely growth stimulators; although, they cannot always be determined analytically because they are often contained in concentrations below the sensitivity threshold of instruments [43,44]. The hormones most commonly detected are cytokinins, auxins, gibberellins and abscisic acid, and, as in other experiments, they also influenced plant height and girth as well as root development in this trial. Traces of ethylene precursors, which promote flowering and fruit ripening in sensitive species, were found in some algae extracts; this effect was also evident in flowers and fruits of Mammillaria. In the past, the positive effects of algae and their extracts on crop productivity have been attributed to the supply of organic matter and, thus, to the improvement in physical, chemical and biological soil fertility [45,46]. However, these effects cannot explain the beneficial action of algae extracts administered to crops in liquid form at extremely low dosages [47]. Recent experimental evidence has shown that liquid algae extracts at low doses manifest positive effects on plant growth, health and crop yield through an action that cannot be attributed to nutrient supply [48,49]. In particular, the biological action of algae extracts has been shown to be due to the presence of carbohydrates, amino acids, vitamins, traces of hormones and hormone-like substances [50]. Studies by Faheed and El Fattah (2008), for example, highlighted the effects of the algae *Chlorella vulgaris* on *Lactuca sativa* L., noting that the presence of the algae accelerated the seed germination process and increased the chlorophyll a and b and carotenoid content [51]. Agwa et al. (2017), using *C. vulgaris* on *Hibiscus esculentus* as a replacement for chemical fertilisers, showed improved plant growth and biomass production [52]. The algae have a high content of primary metabolites, carbohydrates and lipids (55–70% of fresh weight) [53], which certainly interact with plant metabolism, as was also confirmed in this trial. Generally speaking, in *Chlorella* spp., *Chlamydomonas* spp., *Dunaliella* spp. and *Spirulina* spp., carbohydrates can make up 46% of the dry extract, while proteins account for 18–46% of the dry extract. The presence of certain amino acids, such as tryptophan and arginine, in algae extracts, can cause a significant increase in crop growth and yield, as these amino acids are the metabolic precursors of phytohormones. Algae also exhibit an indirect bio-stimulating effect, attributed to their ability to increase and modify the microbial component of the soil, with concomitant effects on nutrient mineralisation as well [54–56]. In experiments on Mammillaria, algae-treated theses showed a significant increase in microbial biomass. Several studies have indeed demonstrated an increase in the soil microbiota following the inoculation of algae, whose prolific production of extracellular polymeric substances served as a carbon source for plants and bacteria in the rhizosphere [57]. The trial emphasised the bio-stimulating capacities of new algae found in Lake Ganzirri and the Venice Lagoon, which had previously not been tested on the growth and productivity of cactus plants, resulting in a significant increase in agronomic parameters, but also in the nutraceutical parameters of the fruits. The latter aspect was certainly influenced by the bioactive compounds of the algae extracts, which stimulated the physiological and biochemical processes of the growing plants [58]. The Mammillaria fruits also showed a better yield than the control and an increase in antioxidant capacity and vitamin C content [59]. Data on *Cucumis sativus* L. also confirmed the same where significant improvements in plant metabolism occurred following treatments with algae such as *Macrocystis pyrifera*, *Bryothamnion triquetrum*, *Ascophyllum nodosum*, *Grammatophora* spp. and *Macrocystis integrifolia*, or on Annurca apple fruits, which showed an increase in total soluble solids (TSS) content, total acidity (TA), pH, flesh consistency and red colouration of the epicarp at harvest stage. The treated fruits also showed a significantly higher content of

total polyphenols in the pulp and a higher concentration of xyloglucoside and floridzin and an increase in flavonols during cold storage [60]. In addition, algae extracts applied to substrates improve the soil microflora by increasing its biological fertility, thus creating a favourable environment for root growth, which increases the exploration capacity of the soil and indirectly influences the bioavailability of nutrients [61]. Indeed, many scientific works have shown how algae extracts can promote crop growth and increase the yield of herbaceous and tree crops and the quality of edible products [34]. The main responses of herbaceous and tree species include increased seed germination rate, root growth, leaf quality, vigour and tolerance to abiotic stresses. Some extracts increase flowering and fruiting and also improve product quality [61].

4. Materials and Methods

Three different macroalgae were sampled and used to prepare the fertiliser: *Hypnea cornuta* (*Rhodophyta*) and *Ulva ohnoi* (*Chlorophyta*) collected from the brackish lake Ganzirri, in Messina, Italy (38°15′28″ N–15°36′37″ E) and *Sargassum muticum* (*Phaeophyceae*) from Venice lagoon, Italy (45°25′42.6″ N–12°19′50.7″ E). After sampling, biomasses were transported in chilled condition after cleaning by washing in fresh water and drying in an oven at 40 °C for 48 h until extraction. For the preparation of Liquid Seaweed Fertilizer (LSF), dried macroalgae were prepared with the modified protocol from Rama (1990) [1]; the process is described in detail in Spagnuolo and Prisa (2021) [2]. In summary, each solution was prepared with 500 mL of distilled water and 25 g of dried seaweed (ratio 1:20 DW/V) at 80 °C for three hours. The residue biomass was removed using a cotton cloth, and the liquid solution gave an SLF that was used in different concentrations in irrigation water on *Mammillaria prolifera* and *Mammillaria glassii*. The plants used were chosen because they produce fruit of edible and medicinal interest and could also be appreciated in the future in view of climate change and the low maintenance they require. To preserve the fertiliser, 1 g/l of citric acid is added and kept at 4 °C until use. The experiments, initiated in September 2021, were conducted in the greenhouses of CREA-OF in Pescia (PT), Tuscany, Italy (43°54′ N 10°41′ E), on *M. prolifera* and *M. glassii*. The plants were grown in ø 12 cm pots, 30 plants per thesis, divided into three replicas of 10 plants each. Plants were fertilised with a controlled-release fertiliser (3 kg m3 Osmocote Pro®, 9–12 months) added to the growing medium of the plants. The experimental groups were as follows:

- Group control (CTRL) (peat 30% + pumice 70%), irrigated with water and substrate previously fertilised;
- Group with algae (AG) (peat 30% + pumice 70%) irrigated with water and substrate previously fertilised, dilution 1:1000 once a week (Kelpak biostimulant, *Ecklonia maxima*, Kelp products International);
- Group with *Hypnea cornuta* (HC) (peat 30% + pumice 70%) irrigated with water and substrate previously fertilised, dilution 1:1000 once a week;
- Group with *Ulva ohnoi* (UO) (peat 30% + pumice 70%) irrigated with water and substrate previously fertilised, dilution 1:1000 once a week;
- Group with *Sargassum muticum* (SM) (peat 30% + pumice 70%) irrigated with water and substrate previously fertilised, dilution 1:1000 once a week.

The concentration and frequency of use in the trial were based on the use of commercial algae, already applied in agriculture. In anticipation of the possible use of these algae and their possible marketing, an attempt was made to compare the protocols and concentrations of use with already tried and tested products. The plants were watered four times a week and cultivated for nine months using an automatic irrigation technology system. On 10 May 2022, plant height and circumference, suckers number, number and length of thorns, vegetative weight, root weight, number of flowers, flowers' life, number and weight of fruits, substrate microbial count, pH, sugar content (g), vitamin C (mg), vitamin A (mg) and vitamin E (mg) concentration were analysed according to Al-Mhanna et al. (2018) [60]. Direct determination of the total microbial count by microscopy of the cells contained in a known volume of sample was performed using counting chambers (Thoma chamber). The

surface of the slide is etched with a grid of squares, with the area of each square known. Determination of viable microbial load after serial decimal dilutions, spatula seeding (1 mL) and plate counting after incubation was performed [61].

Statistics

A randomised block design was used in the experiment and the data obtained were analysed according to the one-way ANOVA to assess whether significant differences existed between the various experimental theses. Then, mean values were separated by LSD multiple-range test ($p = 0.05$). Graphics and statistics were supported by the programs Costas (version 6.451) and Excel (Office 2010).

5. Conclusions

Algae can colonise almost any type of habitat, though most of them live in seas, oceans and fresh waters. However, they can colonise other environments, including deserts, volcanic waters, very acidic or frozen soils, rocks, plants and artificial substrates as well. In the terrestrial environment, algae, especially cyanobacteria, contribute to pools of soil organic matter, either directly or through the secretion of exopolysaccharides and the production of humus-like substances. In addition, they can stimulate the growth of other microflora and microfauna, promote nutrient uptake and participate in organic matter mineralisation processes.

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4.6 Screening on the Presence of Plant Growth Regulators in High Biomass Forming Seaweeds from the Ionian Sea (Mediterranean Sea)

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Screening on the Presence of Plant Growth Regulators in High Biomass Forming Seaweeds from the Ionian Sea (Mediterranean Sea)

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Abstract: The use of seaweed as plant biostimulants is a solution for sustainable agriculture. The present study aims to quantify and compare the presence of plant growth regulators (PGRs) in four genetically labeled macroalgae growing in the Ionian Sea. Species were selected because they produce abundant biomass, disturbing ecological equilibrium and anthropic activities. We measured the content of gibberellic acid (GA_3), kinetin (KN), indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA). The method applied was modified from the literature to obtain simultaneously different PGRs from seaweed biomass in a shorter period of time. Among results, it is notable that *Hypnea corona* Huisman et Petrocelli (*Rhodophyta*) showed higher GA_3 concentration, while in *Spyridia filamentosa* (Wulfen) Harvey (*Rhodophyta*), higher KN, IBA, IAA and ABA contents were recorded. The latter species displayed an interesting profile of PGRs, with an IAA value comparable with that reported in *Ascophyllum nodosum* (Linnaeus) Le Jolis (*Ochrophyta*), which is currently used as a source of plant biostimulants in agriculture. Macroalgae thrive abundantly in nutrient-rich environments, such as anthropized coastal areas affecting human economic activities. Consequently, environmental agencies are forced to dredge algal thalli and discard them as waste. Any use of unwanted biomass as an economic product is highly desirable in the perspective of ecosustainable development.

Keywords: algal biomass; plant biostimulants; HPLC; plant growth regulators; seaweed extracts; sustainable agriculture

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1. Introduction

Increasing drought events, as a consequence of climate change, are causing on a global scale relevant loss in crop yield [1]. Water availability strongly affects plant productivity [2]. Nevertheless, as a likely effect of climate change, an increase in the frequency of extreme events, such as heatwaves, flooding, hurricanes, etc., negatively impacts plant resilience ability, exposing vegetation to higher crop disease and, as a consequence, exacerbating yield reductions [3,4]. Based on alarming forcasts on global warming [5], improving, as much as possible, the resistance and resilience ability as well as the yield of crop plants in such "new" growth conditions is a priority. This challenging task is further needed to meet food demand due to population growth [6]. Optimal mineral nutrition increases plant resilience to different biotic and abiotic stresses, as well as food quality [7]. Nevertheless, chemical fertilization causes high economic and ecological costs [8]. The common practice of improving crop productivity and/or food quality, by using synthetic plant growth

regulators (PGRs) in addition increasing management costs, could be toxic for plants and animals, including humans [9–11].

Plant biostimulants are products that respond to the requirement for agriculture products that are less dependent on synthetic chemicals and at the same time able to provide greater yields and mitigate the effects of climate change, stimulating plant nutrition processes, the tolerance to abiotic stress and crop quality [12–14].

On this view, the use of seaweed is actually a solution for sustainable agriculture because it combines the need to use low-cost but good fertilizers (i.e., it contains minerals) with a source of plant biostimulants. Furthermore, it has the added value of providing a solution to the disposal of unwanted seaweed biomass, which may occur especially in eutrophic environments [15–17].

Seaweeds have been used as an organic fertilizer since ancient times [18]. However, since the 1960s, the seaweed industry has received renewed interest because of an increasing number of studies demonstrating the positive effects of using seaweed extracts on crop productivity and food quality [19].

Seaweeds are mainly applied in organic agriculture due to their biodegradable, non-toxic, non-polluting and non-hazardous effects relative to human and animals [20]. In this regard, extracts obtained by macroalgae represent 30% of the market of plant biostimulants in 2013, 40% of which is absorbed by the European market [21]. Early studies aimed to identify mineral content of seaweed extracts [22,23]. By contrast, in the most recent years, greater attention has been given to PGR contents and other organic compounds frequently present in these natural plant biostimulants.

Macroalgae produce huge biomass in nutrient rich environments, which often are dredged and discarded as waste that do not affect human activities. Such biomass could be a promising source of PGRs in the perspective of eco-sustainable development.

A high and increasing number of studies on the physiological roles of phytohormones in terrestrial plants improve our understanding of their effects and interactions on plant growth and productivity. Conversely, the same knowledge is lacking for seaweeds [24]. This gap may negatively impact the advantages of using seaweed extracts on crops. On this view, verifying and quantifying the presence of plant biostimulant products in the algae are prerequisites to any project related to their physiological involvement.

The present study aims to quantify and compare the presence of some PGRs in four different macroalgae growing in the Ionian Sea (Taranto, Italy), i.e., two *Rhodophyta* (*Spyridia filamentosa* (Wulfen) Harvey and *Hypnea corona* Huisman et Petrocelli) and two *Chlorophyta* (*Chaetomorpha linum* (O.F. Müller) Kützing and *Ulva lacinulata* (Kützing) Wittrock). The four algal species were selected because they produce abundant biomass in the collection site, disturbing both ecological equilibrium and anthropic economic activities.

In detail, we measured the content of gibberellic acid (GA_3), kinetin (KN) indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) as the main representatives of PGRs as a screening prerequisite for further tests to evaluate their agronomic effects.

2. Materials and Methods

2.1. Collection of Algae

Samples of *Spyridia filamentosa* (Wulfen) Harvey, *Hypnea corona* Huisman et Petrocelli (*Rhodophyta*), *Chaetomorpha linum* (O.F. Müller) Kützing and *Ulva lacinulata* (Kützing) Wittrock (*Chlorophyta*) were collected from Taranto (Italy, Ionian Sea) (Table 1).

Species names and phylum attributions are in accordance with algaebase.org [25]. All species used in the present investigation have isomorphic life cycles, with alternating gametophytic and sporophytic phases, which thrive in mixed populations and are distinguishable only by fine reproductive aspects by trained experts. In the perspective of potential economic exploitation, we decided to use natural populations and to process mixed haploid and diploid batches.

Table 1. List of the algal samples used in this study.

	Species	Voucher ID
Rhodophyta	*Hypnea corona* Huisman et Petrocelli	PhL705
	Spyridia filamentosa (Wulfen) Harvey	PhL706
Chlorophyta	*Chaetomorpha linum* (O.F. Müller) Kützing	PhL707
	Ulva lacinulata (Kützing) Wittrock	PhL708

After collection, samples were immediately washed with seawater to remove possible debris, adhering sand particles and epiphytes and then transported to the laboratory in plastic bags at low temperatures and washed with tap water to remove surface salt. From each sample, a portion was dried in silica gel and stored at −20 °C for DNA barcoding identification, and the remaining portions were dried for 72 h in an oven at 65 °C. Then, the samples were powdered by an electric grinder stored in plastic bags at 4 °C until they were analyzed.

DNA barcoding identification was performed according to protocols described in Miladi et al. [26] and Manghisi et al. [27]. Selected barcodes were COI-5P for *Rhodophyta*, tufA for *U. lacinulata* and LSU D2/D3 for *C. linum* [28]. DNA sequencing reactions were performed by an external company (Macrogen Europe, Amsterdam, The Netherlands). Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd., South Brisbane, QLD, Australia), and species attributions were performed by the identification engine in BOLD Systems (www.boldsystems.org, accessed on 6 January 2022) and the BLAST tool at the National Center for Biotechnology Information (Bethesda, MD, USA, blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 January 2022).

2.2. Preparation of Standard Solutions

Standard substances (purity > 98%) of gibberellic acid (GA_3), indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) were purchased from OlChemIm s.r.o. (Olomouc, Czech Republic). Kinetin (KN) was purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical HPLC-grade methanol (MeOH) and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Distilled water was deionized in an Elga Veolia Purelab ultra-pure water system (High Wycombe, UK).

The standard compounds were dissolved in MeOH:H_2O (50:50 v/v) at a stock concentration of 1000 µg/mL and stored at 4 °C. Working standard solutions were obtained by diluting them with MeOH: H_2O (50:50 v/v) prior to use. All solvents were ultrasonified for 30 min (Sonica, Soltec, Japan) before use.

2.3. Sample Preparation

An aliquot (0.5 g) of each powdered sample was infused in a 4 mL MeOH:H_2O solution (80:20 v/v) with 1 mmol/L of citric acid as the antioxidant. Solutions were sonicated for 15 min and placed in infusion for 3 days at 4 °C. The samples were transferred in 15 mL vials and centrifuged at 7000 rpm for 30 min at 4 °C. Then, the supernatants were collected and 1 mL of MeOH was added. After 1 h, the samples were centrifuged for an additional 15 min at 7000 rpm at 4 °C, filtered with 0.2 µm in diameter syringe filter and diluted with ultra-pure water (1:5). No purification steps were performed to speed up the protocol.

2.4. HPLC Set-Up

Chromatographic runs were carried out on a Beckman Coulter 126 binary pumps HPLC system with the detector Beckman Coulter 166 UV/VIS system (Brea, CA, USA). Karat 32 ver. 8.0 software was employed for instrument control and data acquisition. Data analyses were accomplished by in-house Octave script. Starlab scientific (Xi'an, China) XChroma universal-C18 column (5 µm, 120 Å, 4.6 × 250 mm) was used as the separation channel.

The mobile phase was composed of MeOH:H_2O (70:30, v/v), both acidified with acetic acid 0.5%, and the flow rate was 1.0 mL/min. The UV/Vis detector was set to 280 nm. The injection volume was 20 µL for each analysis using IDEX corp. Rheodyne 7125 valve (Lake Forest, CA, USA). All samples were analyzed in 3 repetitions. The results are presented as mean ± standard deviation.

2.5. Method Validation

The HPLC method was validated by the evaluation of the variation of retention times and peak area for analytes, performing calibration curves, limit of detection (LOD), limit of quantification (LOQ) and accuracy.

The analytical performances and the calibration curve are summarized in Table 2. The limit of detection (LOD), obtained by evaluation of signal to noise ratio, ranges between 5 µg/mL for GA_3 and 0.2 µg/mL for KN, IAA and ABA. The calibration curves showed a linear trend, and the reliability of measurements were confirmed by intra- and inter-day analysis, and the standard deviations are less than 2%. The retention time of the standards was 4.6, 5.3, 7.5, 9.2 and 12.3 min for GA_3, KN, IAA, ABA and IBA, respectively.

Table 2. Analytical performance data for major endogenetic plant growth regulators. R^2: correlation coefficient. LOD: limit of detection.

Analyte	Range (µg/mL)	Equations	R^2	LOD (µg/mL)	Degree of Freedom
GA_3	10–1000	y = 0.0433x + 0.0013	0.9896	5	6
KN	0.1–10	y = 3.9094x − 0.0008	0.9937	0.2	6
IAA	0.1–10	y = 0.5829x + 0.0006	0.9967	0.2	6
ABA	0.1–10	y = 1.8133x − 0.0004	0.9985	0.2	6
IBA	0.1–10	y = 0.4685x + 0.0007	0.9965	1.8	6

The standard solutions were found to be stable for months and stored at −25 °C; any variations on the value of response function were observed in the chromatogram recorded.

3. Results and Discussion

Seaweeds are known to produce plant growth regulators (PGRs), similarly to land plants [29]. Their effects include the response to various developmental and physiological processes and provide support to overcome abiotic and biotic stresses [30]. Recently, the attention of researchers pointed to the detection and quantification of different PGRs in seaweeds with the aim of agronomic applications [30].

The method applied in the present work was modified from Gupta et al. [31] in order to obtain simultaneously different PGRs from seaweed biomass but in a shorter period time. The separation of PGRs was performed by simplifying the extraction process, with a complete run performed in 18 min. The most significant modification was the lack of purification of the extracts in order to make the protocol faster and cheaper in the framework of applicative exploitation.

Overall, the *Rhodophyta* species analyzed in the present work showed a higher content of PGRs than the analyzed *Chlorophyta* (Table 3, HPLC chromatograms in Supplementary Materials). These data, however, cannot be drawn as a general conclusion that *Rhodophyta* as a whole have a higher content of PGRs than *Chlorophyta*, as the present results regard a limited taxonomic span. More research is needed to achieve a general framework.

In detail, *Hypnea corona* Huisman et Petrocelli showed higher GA_3 concentration, while in *Spyridia filamentosa* (Wulfen) Harvey, higher KN, IBA, IAA and ABA contents were recorded. The latter species displayed an interesting profile of PGRs, with an IAA value comparable with that reported in *Ascophyllum nodosum* (Linnaeus) Le Jolis (*Ochrophyta*) [32], which is currently used as a source of plant biostimulants in agriculture [33–36].

Table 3. Gibberellic acid (GA_3), kinetin (KN) indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) contents as estimated by HPLC-UV in extracts of the four investigated seaweed extracts. Values are presented as means of three measurements with standard deviations. LOD: limit of detection. LOQ: limit of quantification.

Species	GA_3 (µg/mL)	KN (µg/mL)	IAA (µg/mL)	ABA (µg/mL)	IBA (µg/mL)
Hypnea corona Huisman et Petrocelli	1038.00 ± 2.00	0.57 ± 0.07	6.70 ± 0.30	1.10 ± 0.40	LOQ
Spryridia filamentosa (Wulfen) Harvey	6.30 ± 0.10	1.70 ± 0.20	63.60 ± 0.50	8.40 ± 0.90	17.90 ± 0.00
Chaetomorpha linum (O.F. Müller) Kützing	5.40 ± 0.40	0.31 ± 0.05	LOD	0	LOQ
Ulva lacinulata (Kützing) Wittrock	0	0.48 ± 0.06	2.30 ± 0.10	0.72 ± 0.02	LOQ

In *Chaetomorpha linum* (O.F. Müller) Kützing, no IAA, IBA and ABA were present. *Ulva lacinulata* (Kützing) Wittrock showed a concentration of KN and ABA similar to *H. corona* but lower than *S. filamentosa* and no GA_3 content.

The presence of PGRs in seaweeds has been already reported in several studies [31,37,38]. However, the occurrence and the roles of these molecules on seaweeds physiology are still not clear [39–41].

Nevertheless, the literature data strongly suggest that PGRs in seaweeds are not a mere result of some metabolic processes but may have specific physiological relevance on their growth as a response to environmental stimuli [37]. In accordance, it has been shown that changes in PGR concentration occur in response to abiotic stress in different seaweed species [38,39,42]. Moreover, different studies highlighted similar roles of PGRs in terrestrial plants versus seaweeds species. As an example, IAA affects the embryo development of *Brassica juncea* (Linnaeus) Czernajew (*Magnoliophyta*) as well as of *Fucus distichus* Linnaeus (*Ochrophyta*) germlings [43,44]. Similarly to terrestrial plants, ethylene promoted chlorophyll degradation in *Ulva intestinalis* Linnaeus [45] and affected the maturation of reproductive structures in *Pterocladiella capillacea* (S.G. Gmelin) Bornet (*Rhodophyta*) [46]. ABA, a phytohormone generally associated to several stress responses in terrestrial plants [47,48], is involved in the coping oxidative stress of intertidal seaweed species [49]. Stirk et al. [50] recorded higher ABA content in *Ulva lactuca* Linnaeus (as *Ulva fasciata* Delile) versus *Dictyota humifusa* Hörnig, Schnetter et Coppejans (*Ochrophyta*), likely as a result of a stronger environmental stress. Brassinosteroided-mediated ABA synthesis occurred in response to thermal stress in brassicacean *Chorispora bungeana* Fischer et C.A. Mey (*Magnoliophyta*) as well as in green alga *Chlorella vulgaris* Beijerinck (*Chlorophyta*) [51,52]. Furthermore, in terrestrial plants and frequently but not exclusively in response to biotic and abiotic stresses, ABA plays a key role in different developmental processes, including seed germination, root and shoot development and photosynthesis inhibition [53–58]. Likewise, ABA impacted plant growth in *Laminaria* J.V. Lamouroux spp. (*Ochrophyta*), inhibited the growth of sporophyte, but it stimulated sorus formation in *Saccharina japonica* (Areschoug) C.E. Lane, C. Mayes, Druehl et G.W. Saunders (as *Laminaria japonica* Areschoug) and constrained the photosynthesis of *Fucus vesiculosus* Linnaeus embryos [59–61].

4. Conclusions

In the present study, PGRs were recorded in four macroalgal species belonging to *Rhodophyta* and *Chlorophyta*. Their physiological role was not investigated, which was out of the scope of our research. However, it could be speculated that PGRs in algae affect their growth and response to environmental factors, similarly to terrestrial plants in accordance with the literature (see above). Even if understanding the physiological functions of PGRs in algae is very interesting for fundamental physiological research, it is not essential for seaweed industrial exploitation, including the use of algal biomass as a source of plant biostimulants in the framework of sustainable agriculture. Further studies are needed to evaluate the effective applications of algal PGRs in agriculture, testing both protocols to produce algal plant biostimulants and application strategies on the growth of agronomic species in the field.

The novelty of the present research relies not only in reporting the presence of PGRs in algae as a whole but also on suggesting the use of unwanted biomasses and testing them for the presence of PGRs for the purpose of their exploitation in agriculture. Moreover, due to the large genetic and consequently metabolic diversity of macroalgae, the aim is to investigate more taxa as a source of PGRs from diverse geographical sites and add useful data in the knowledge of algal physiology.

Macroalgal identification at the species level is a complex task, which need the involvement of skilled taxonomists. In the perspective of industrial exploitation, we recommended that genetic labeling should be used. The official DNA barcode is a prompt and effective tool, which proved to be useful in applied research, e.g., [62–64].

Macroalgae thrive abundantly in nutrient rich environments, such as anthropized coastal areas. Such biomass affects human economic activities, disturbing navigation, aquaculture and tourism, as examples. As a consequence, environmental agencies are forced to dredge algal thalli and discard them as a waste. Any use of unwanted biomass as an economic product is highly desirable in the perspective of eco-sustainable development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14073914/s1, Figure S1: *Hypnea corona* Huisman et Petrocelli, HPLC chromatogram. Minutes reported in decimal divisions; Figure S2: *Spyridia filamentosa* (Wulfen) Harvey, HPLC chromatogram. Minutes reported in decimal divisions; Figure S3: *Chaetomorpha linum* (O.F. Müller) Kützing, HPLC chromatogram. Minutes reported in decimal divisions; Figure S4: *Ulva lacinulata* (Kützing) Wittrock, HPLC chromatogram. Minutes reported in decimal divisions.

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5 Stage and consulting

During the doctoral activity collaborations were established and developed with academic, public and corporate research centres. The research activities led to a fruitful collaboration as the first focus of bridging the gap between academia and companies and bringing the research streams to an increased level of multidisciplinary.

Certain uses, especially in the agricultural, nutritional and cosmetic fields, have led to a greater knowledge of the algae that can be grown in Europe but, above all, in Italy.

Research activities listed hereafter have not been published yet and some of them are protected by non-disclosure agreements established with companies.

Danish Shellfish Centre, Nykøbing Mors, Technical University of Denmark (DTU) (Denmark)

Tetrasporophytes of *Asparagopsis armata* and *A. taxiformis,* used as a food additive in the livestock diet to reduce methane emissions, were cultivated to improve and refine the growth parameters in indoor systems. The growth parameters considered were light (photoperiod and light intensity), temperature, stock density, fragmentation, and addition of CO_2 by air insufflation, in order to improve biomass and to preserve halogenated compounds.

Ciencias Ambientales Centro EULA de Ciencias Ambientales, University of Concepción (Chile)

Was evaluated the content of polyphenols extracted and their antioxidants effect from European algae species (*Sargassum muticum, Chaetomorpha aerea, Dictyota* sp., *Saccharina latissima, Ulva ohnoi* and *Hypnea cornuta*). Moreover, antibacterial activity was tested against both pathogens and environmental bacteria.

ApuliaKundi srl (Italy)

Setting up of a pilot scale cultivation of *Pyropia* spp. evaluating the effects of growth parameters (temperature, nutrients, intensity and light period), focusing on vitamin B_{12} and Taurine thallus contents.

South Agro srl (Italy)

Setting up of a pilot scale cultivation of species of *Ulva* present in the Mediterranean Sea with the aim of obtain biofertilizers. Setting up of a manual for the taxonomic identification of macroalgae from Europe.

HS srl (Italy) and ForFoods srl (Italy)

Setting up of pilot scale cultivations of *Ulva* spp. and *Graciliaria* spp. Refinement of extraction protocols of metabolites from algae, in particular alcoholic and aqueous extracts and evaluation of different extractions from in the frame of biorefinery.

Department Veterinary Sciences, University of Messina (Italy)

We evaluated the antimicrobial activity of *Asparagopsis* spp. ethanolic extracts against different *Listeria monocytogenes* strains. Furthermore, the Minimal Inhibiting Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were evaluated by microdilution method.

6 Conclusion

The request of seaweed and derivated products has increased worldwide, as well as the interest of stakeholders in different types of macroalgae that can meet the various needs of the economy. Seaweed biomass import and export is increasing in Europe; this leads to a scarcity of raw material with a consequent increase in production and accordingly the demand of expertise and know-out it concerns. To be able to meet these market requests, it is necessary to build a network between academia and companies. The production must necessarily maintain high quality standards, which also concerns the need to standardize the management plants in all aspects, including biosecurity. Macroalgae have intrinsic eco-sustainability properties as they do not require fresh water for their growth, it is not necessary to use fertile soil which can be reserved for the cultivation of terrestrial plants, for the growth they use CO_2 and chemical compounds containing nitrogen and phosphorus which can cause eutrophication. In fact, many algae can grow in IMTA crops and with industrial wastewater. Algae thrive abundantly in dystrophic environments and large quantities of biomass negatively interfere with the growth of other organisms. Any use of these natural populations, especially of invasive species, would involve the valorisation of unwanted biomass, commonly treated as waste, converting it into a precious resource. The ecological transition based on production without waste of materials and energy is the fundamental principle for facing the future development of global economic systems. In a circular

economy context, the valorisation of waste as a raw material for innovative production processes becomes a priority objective for safeguarding the environment. In basins where the proliferation of algal species becomes an obstacle for economic activities and for the quality of the environment, scientific research should be directed towards the development of strategies that exploit this biomass. From a biorefinery point of view, the algal biomass waste can become raw material for the production of bioactive compounds with high commercial value, such as antimicrobials and phycocolloids, and also use of eco-sustainable thermochemical conversion processes. Waste algal biomass, in fact, thanks to its chemical composition, rich in carbon, nitrogen and other nutrients, is a promising source of materials, with a view to eco-sustainable development. All of this can only be done through an in-depth knowledge of the taxonomy and physiology of used seaweeds, starting from the taxonomic identification of the algae. From the perspective of economic exploitation, the use of genetic labelling (DNA barcoding) is therefore desirable, which provides a permanent floristic archive of macrophytes with a view to characterizing the natural environment, as well as the traceability of the final product. In terms of remediation and mitigation of anthropic pressure, the removal of unwanted algal biomass certainly has a positive effect in confined ecosystems, regulating the fluctuations of the trophic state of the system, contrasting dystrophic phenomena and potential anoxic crises and, ultimately,

contributing to the mitigation of climate change. Adequate monitoring of the ecosystem quality of the water bodies involved is necessary to evaluate the benefits that may be achieved. The production of macroalgae meets all the environmental objectives referred to in Regulation 2020/852 of the European Parliament and of the Council of 18 June 2020 concerning the establishment of a framework that favours sustainable investments and amending Regulation (EU) 2019/2088. In detail, it aims to contribute to the transition towards a circular economy through the sustainable use of biological resources, the protection of biodiversity and ecosystems through the recovery and conversion of algal biomass to produce systems aimed at reducing pollution.

In the present thesis, attention was paid to the coastal lakes of Ganzirri and Faro (Messina, Italy), both susceptible to dystrophic phenomena, within the naturally oriented reserve of Cape Peloro (ZSC ITA030008, D.M. 21/12/2015), which represent an ideal environment from an ecological perspective as well as for its ethnoanthropological richness.

From the point of view of biosustainability and circular economy, extracts from macroalgae should respect these principles, aiming to lower the use of chemicals, optimize energy consumption of processes, while maintaining high quality standards and, if possible, increasing product yield.