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Enhancing ex situ cultivation of Mediterranean Fucales: species-specific responses of *Gongolaria barbata* and *Ericaria crinita* seedlings to algal extracts

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ABSTRACT

Mediterranean brown algal forests, dominated by *Cystoseira sensu lato* species, are undergoing widespread decline due to the cumulative effects of anthropogenic pressure and climate-related stressors. Restoration efforts increasingly rely on ex situ cultivation and outplanting of seedlings, yet early developmental stages often suffer from low survival and growth rates. This study investigated the potential of algal extracts to enhance the seedling development and survival in two canopy-forming species, *Gongolaria barbata* and *Ericaria crinita*. We tested extracts from a cyanobacterium (*Trichormus variabilis*), two microalgae (*Desmodesmus* sp. and *Cylindrotheca closterium),* and a commercial macroalgal formulation (AlgatronCifo®) at varying concentrations under controlled mesocosm conditions. Seedling performance was significantly influenced by extract type, and target species identity. Notably, a low-concentration *Desmodesmus* sp. extract (0.07 mg mL^{-1}) improved survival and growth, whereas *T. variabilis* exerted an inhibitory effect on *G. barbata*. AlgatronCifo® did not outperform *Desmodesmus* sp. extract in promoting seedling development. These findings suggest that specific extracts from green microalgae could improve protocols for the early stages of restoration, offering a scalable tool for rehabilitating degraded marine forests. However, the results underscore the importance of species-specific optimization and the need for in situ validation of biostimulant-based restoration approaches.

1. Introduction

Mediterranean macroalgal forests, dominated by canopy forming brown algae of the order Fucales (primarily the *Cystoseira sensu lato* complex), form structurally complex habitats that are functionally analogous to kelp forests in temperate and cold oceans. These foundational ecosystems support high biodiversity, providing food, shelter and critical ecosystem services, while acting as significant carbon sinks (Cheminée et al., 2017; Piazzi et al., 2018; De La Fuente et al., 2019a; Peleg et al., 2020; Sant and Ballesteros, 2021; Steneck et al., 2002). However, these vital ecosystems have undergone severe decline, with several populations becoming fragmented or locally extinct, often replaced by ephemeral and filamentous taxa (Thibaut et al., 2015; Mariani et al., 2019; Bernal-Ibáñez et al., 2021; Orlando-Bonaca et al., 2021a). This degradation stems from multiple anthropogenic pressures including habitat destruction, increased sediment resuspension, chemical pollution, and overgrazing by herbivores (Mangialajo et al., 2008; Orlando-Bonaca and Rotter, 2018; Piazzi and Ceccherelli, 2019; Orfanidis et al., 2021). Climate change has exacerbated these impacts, particularly through the increasing frequency and intensity of marine

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heatwaves (Bevilacqua et al., 2019; Verdura et al., 2021; Martínez et al., 2023).

The natural recovery potential of these damaged ecosystems is severely limited even if the primary stressors have been removed, with recolonization being rare due to propagule dispersal limitations and lack of source populations (Clayton, 1990; Galobart et al., 2023), with only isolated cases of recovery documented (Perkol-Finkel and Airoldi, 2010; Iveša et al., 2016; Medrano et al., 2020). Consequently, human-assisted restoration has proven to be essential (Cebrian et al., 2021), as recognized by international policies like the EU Nature Restoration Act (EC, 2024).

Significant progress has been made in developing restoration techniques for *Cystoseira s.l.* species over the past fifteen years. Collaborative research efforts have yielded diverse protocols encompassing both in situ and ex situ approaches (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019b; Medrano et al., 2020; Orlando-Bonaca et al., 2022; Lokovšek et al., 2023). Although still implemented at small to medium scales, these restoration measures currently represent the most viable strategy to reverse the loss of *Cystoseira s.l.* forests in the Mediterranean (Smith et al., 2023).

In the northern Adriatic basin, where populations from the order Fucales have declined sharply (Falace et al., 2010, 2024; Orlando-Bonaca and Rotter, 2018; Iveša et al., 2022) ex situ cultivation approaches have been used in various restoration initiatives (Savonitto et al., 2021; Orlando-Bonaca et al., 2021b, 2022; Lokovšek et al., 2023, 2024). Kaleb et al. (2023) demonstrated that the commercial algal biostimulant AlgatronCifo® (from *Macrocystis pyrifera* (Linnaeus) C. Agardh (Phaeophyceae) can improve physiological performance and induce fertility in adult thalli of *Gongolaria barbata* (Stackhouse) Kuntze. This finding aligns with the well-established use of algal biostimulants in terrestrial agriculture and emerging applications in seaweed farming (Nanda et al., 2022; Jiksing et al., 2022).

Algal biostimulants (as defined by EU Regulation, 2019/1009 (see European Union, 2019)) are known to promote nutrient uptake and assimilation, increase chlorophyll content and photosynthetic activity, and improve resilience to abiotic stressors (Calvo et al., 2014). Microalgae and cyanobacteria offer particular advantages as biostimulants sources, due to their efficient cultivation and bioactive compound production (Sánchez-Quintero et al., 2023), including phytohormones, amino acids, antimicrobial metabolites (Parwani et al., 2021; Rossi and De Philippis, 2015) and ROS-scavenging antioxidants (Abinandan et al., 2019). Extracts have enhanced stress tolerance of terrestrial plants (Puglisi et al., 2020), but marine applications remain untapped.

Notably, microalgae exhibit substantial biocontrol potential against phytopathogens through bioactive metabolites (Grabowski et al., 2024), thus addressing a critical constraint in ex situ restoration: microbial contamination during early developmental stages (e.g., Savonitto et al., 2021; Malfatti et al., 2023). The ability of algal compounds to suppress common pathogens while potentially enhancing beneficial microbial associations addresses a critical bottleneck in seedling cultivation (Malfatti et al., 2023).

Despite these advances, the application of microalgal- and cyanobacterial-derived biostimulants in marine habitat restoration remains largely unexplored. Their demonstrated capacity to enhance growth (e.g., frond elongation, rhizoid development), stress resistance (e.g., to temperature fluctuations, oxidative stress), and microbial symbiosis, combined with cost-effective scalability in photobioreactor systems, positions them as transformative tools for optimizing ex situ cultivation protocols in macroalgal restoration programs.

The present study addresses this critical knowledge gap through two primary objectives:

1. Quantifying the species-specific responses of *Gongolaria barbata* and *Ericaria crinita* (Duby) Molinari & Guiry to microalgal and macroalgal extracts, measuring the effects on embryo development, seedling growth, and survival;
2. Evaluating practical applications for enhancing ex situ restoration of Mediterranean brown algal forests.

By investigating these novel biostimulant approaches, this work aims to develop more effective and scalable methods to restore these vital but threatened marine ecosystems.

2. Material and methods

2.1. Microalgae extracts production and characterization

The study utilized three microalgal strains selected for their documented biostimulant properties and ecological relevance: the cyanobacterium *Trichormus variabilis* (VRUC168), the green microalga *Desmodesmus* sp. (VRUC281), and the diatom *Cylindrotheca closterium* (VRUC291). These strains originate from the Vergata Rome University Culture Collection (VRUC), where they are maintained in batch stock cultures under controlled conditions (18 °C, 30 μmol photons m^{-2} s^{-1}, 12:12 light:dark cycle) using BG11.0 medium for *T. variabilis* and BG11 medium for *Desmodesmus* sp. and *C. closterium*.

The selected strains offer complementary biostimulant properties rooted in their distinct biochemical profiles. *Trichormus variabilis* enhances plant growth and antioxidant defenses under abiotic stress through the production of exopolysaccharides, phytohormones, and nitrogen-fixing metabolites, as demonstrated in crops like rice and maize (Bauenova et al., 2024). *Desmodesmus* species show robust biostimulant activity across plant systems, promoting germination, seedling vigor and nutrient uptake via their high content of cytokinins (particularly zeatin), amino acids and antioxidant compounds (Pereira et al., 2021; Perkol-Finkel and Airoldi, 2010; Araújo et al., 2023). *Cylindrotheca closterium* has antioxidant and anti-inflammatory potential, with transcriptomic studies confirming its biosynthetic capacity for bioactive metabolites (Lauritano et al., 2019; 2020). This phylogenetic and functional diversity provides a comprehensive biochemical foundation for developing nature-based solutions to improve the success of macroalgal restoration and ecosystem resilience.

The strains were mass cultivated indoors in photobioreactors of different geometry and capacity at 24 °C, with an irradiance of 30 μmol photons m^{-2} s^{-1} and an L/D cycle of 12:12 in semi-continuous mode. Harvesting of biomass involved settling and centrifugation (4500×*g* for 10 min) after the stationary phase, approximately after 4 weeks of growth. Specifically, *T. variabilis* was intensively cultured in an 8 L column photobioreactor, *Desmodesmus* sp. in a 2 L Erlenmeyer flask and *C. closterium* in a 6 L spherical system, with all cultures exposed to air insufflation for mixing. The harvested biomass was finally lyophilised (Telstar LyoQuest) for further experiments.

For biomass extraction, 3 g of the freeze-dried biomass samples were grinded and placed in a 1 L Erlenmeyer flask and 300 mL bidistilled water was added. Then the biomass was resuspended and treated for 20 min under high pressure (1 atm) and at high temperature (121 °C). After cooling, the biomass was centrifuged at 4500 g for 10 min and the supernatants, referred to as 'green extracts', were collected in 50 mL Falcon tubes, stored at −20 °C and freeze-dried.

2.1.1. Biochemical characterization of the extracts

The total nitrogen (TN) and phosphorus (TP) content of the extracts was quantified using persulfate digestion (Langner and Hendrix, 1982). The digestion reagent consisted of 50 g L^{-1} potassium persulfate ($K_2S_2O_8$), 30 g L^{-1} boric acid (H_3BO_3), and 15 g L^{-1} sodium hydroxide (NaOH). 10 mL of the digestion reagent was added to 5 mg of extracts in 100 mL Erlenmeyer flasks, which were then sealed with aluminum foil and subjected to autoclave digestion (121 °C for 30 min at 1 atm). During digestion, nitrogen- and phosphorus-containing compounds were oxidized to nitrate (NO_3^-) and orthophosphate (PO_4^{3-}), respectively, due to the alkaline environment created by the reagent (pH ≈ 9.7). The subsequent decomposition of potassium persulfate led to a

drop in the pH value (≈1.5), promoting phosphorus oxidation.

After cooling to room temperature, the TN concentration was determined spectrophotometrically by recording the absorbance at wavelength (λ) of 220 nm using quartz cuvettes. The concentration of orthophosphates in the digested sample (corresponding to TP) was determined using the colorimetric molybdenum blue method (Murphy and Riley, 1962). The reagent was freshly prepared before each analysis and added in the following sequence:

- 100 mL sulfuric acid (136 mL L^{-1}),
- 250 mL ammonium molybdate (40 g L^{-1}),
- 100 mL potassium antimonyl tartrate (3 g L^{-1})
- 50 mL ascorbic acid (9 g L^{-1})

The reaction between orthophosphates, ammonium molybdate, and potassium antimonyl tartrate resulted in the formation of a phosphomolybdate complex, which was subsequently reduced by L-ascorbic acid to form the molybdenum blue complex, which has a maximum absorbance at a wavelength of 882 nm. A volume of 2 mL of the reagent was added to the samples and calibration standards. After the colorimetric reaction, the TP concentration was measured spectrophotometrically at 882 nm using plastic cuvettes. The calibration curves were prepared with known concentrations of KNO_3 and KH_2PO_4. Absorbance readings were made using an ONDA UV-30 Scan spectrophotometer.

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method reagent, as described in Di Marco et al. (2013). Briefly, 10 mg of each sample was extracted in 4 mL of absolute methanol (CH_3OH) overnight at room temperature with continuous stirring (orbital shaker; 110 rpm). The suspensions were then centrifuged (5000×*g*, 20 min), and 20 μL of each supernatant was transferred to a Greiner Multiwell plate, followed by the addition of 80 μL Folin reagent diluted in ultra-pure H_2O (1:10 *v/v*). The mixture was incubated for 8 min before 20 μL of 20 % sodium carbonate solution was added. After 2-h of incubation, the absorbance was measured at 765 nm using a Tecan Spark fluorescence microplate reader (Tecan, Switzerland). The results were expressed as μg gallic acid equivalents (GAE) per mg fresh weight (mg FW).

2.2. Study sites and sample collection

Fertile apices of *Gongolaria barbata* were collected by SCUBA divers in April–May 2024 in Izola, Slovenia (45.5436°N, 13.6764°E) at 1–2 m depth, harvesting ≤10 % of reproductive tissue per individual to minimize population impact. *Ericaria crinita* samples were collected in June 2024 from Bijela Uvala, Croatia (45.1868°N, 13.5890°E) at 0.5–2 m depth. All specimens were transported to the Marine Biology Station Piran (MBSP, Slovenia) and the Center for Marine Research Rovinj (CMRR, Croatia) within 4 h of collection in a dark, temperature-controlled environment (4 °C). Upon arrival at the MBSP and CMRR nursery facilities, the apical parts of the thalli of *G. barbata* and *E. crinita* were rinsed with filtered seawater to remove epibionts and then stored at 4 °C for 24 h to promote gamete release. The fertile material was then cultured in a thermostatic chamber in three different experimental setups. The cultivation process followed the protocol of Falace et al. (2018) and De La Fuente et al. (2019b), which was further improved in Lokovšek et al. (2023).

2.3. Experimental designs

The sampling sites, brown algae species and experimental purposes are summarized in Table 1. The three experimental setups, and the concentrations of the algal extracts tested are explained in detail in subsections 2.3.1-2.3.3.

Table 1
Overview of sampling sites, species and time periods, experimental purposes, nursery facilities, types of algal extracts tested, and concentrations used in ex situ experiments conducted in 2024.

Sampling site	Sampled species	Sampling period	Experimental purposes	Nursery facility	Algal extracts tested	Concentrations
Izola (Slovenia)	*Gongolaria barbata*	early April 2024	1st experiment - Preliminary screening of microalgal extracts on early developmental stages	MBSP	*Trichormus variabilis, Cylindrotheca closterium, Desmodesmus* sp.	for all: 2.25 mg mL^{-1} and 4.50 mg mL^{-1}
Izola (Slovenia)	*Gongolaria barbata*	mid-April 2024	2nd experiment - Selection of the most effective microalgal extract and concentration on early developmental stages	MBSP	*Trichormus variabilis, Cylindrotheca closterium, Desmodesmus* sp.	for all: 1.2 mg mL^{-1}, 0.6 mg mL^{-1}, 0.3 mg mL^{-1}, 0.15 mg mL^{-1} and 0.07 mg mL^{-1}
Izola (Slovenia)	*Gongolaria barbata*	mid-May 2024	3rd experiment - Comparative assessment of microalgal vs. macroalgal extracts on early developmental stages	MBSP	*Desmodesmus* sp. (D), AlgatronCifo® (A)	0.07 mg mL^{-1} (D) and 4.5 mL L^{-1} (A)
Rovinj (Croatia)	*Ericaria crinita*	early June 2024	3rd experiment - Comparative assessment of microalgal vs. macroalgal extracts on early developmental stages	CMRR	*Desmodesmus* sp. (D), AlgatronCifo® (A)	0.07 mg mL^{-1} (D) and 4.5 mL L^{-1} (A)

2.3.1. First screenings of microalgal extracts and concentrations on G. barbata germlings

The first experimental trial was designed to evaluate the effects of three algal extracts (*Trichormus variabilis*, *Desmodesmus* sp., and *Cylindrotheca closterium*) on early developmental stages of *G. barbata* germlings. Based on previous studies of macroalgal cultivation and biostimulant applications (Guzmán-Murillo et al., 2013; Amer et al., 2019; Santini et al., 2021), we tested each extract at two concentrations (2.25 mg mL^{-1} and 4.50 mg mL^{-1}).

The experiment was performed in a temperature- and light-controlled room using 40 mL Petri dishes. Ambient conditions were maintained at 17 °C with a 15:9 h photoperiod (L:D) according to established protocols (Orlando-Bonaca et al., 2021b). Illumination was provided by four 36W Osram Fluora fluorescent tubes (120 cm in length) delivering an irradiance of approximately 140 μmol photons m^{-2} s^{-1} to the culture surface.

The experimental setup (Fig. 1) comprised triplicate dishes for each extract-concentration combination (18 treatment dishes in total) plus three control dishes containing Von Stosch enriched medium prepared with UVC-sterilized seawater (VS). The VS control followed methodologies from multiple previous studies of *G. barbata* cultivation (Falace et al., 2018; De La Fuente et al., 2019b; Savonitto et al., 2021; Orlando-Bonaca et al., 2021b, 2022; Lokovšek et al., 2023).

Five apices of *G. barbata* (approx. 3 cm long), each bearing mature receptacles, were placed in each Petri dish and left undisturbed for 48 h to allow gamete release and fertilization. The apical segments were then carefully removed so as not to disturb zygote development. Zygote development and growth, and survival rate was monitored by photographing a fixed area of 0.12 cm^2 in each dish at 24–48 h intervals using a stereomicroscope equipped with a high-resolution digital camera. This photographic documentation continued throughout the early developmental phase to track treatment effects on germination success and early growth.

2.3.2. Evaluation of the concentration effects of the different microalgal extracts on G. barbata seedlings

Based on the results of the preliminary screening, we conducted a second experiment to identify the most effective algal extract and determine its optimal concentration for promoting the development of *G. barbata* seedlings. This refined experimental approach utilized smaller culture volumes (10 mL Petri dishes) and a series of dilutions prepared from a 4.5 mg mL^{-1} stock solution (Fig. 2).

The experimental setup comprised duplicate treatments (n = 2) for each extract-concentration combination, with UVC-sterilized seawater serving as a control medium. For each replicate, we placed three fertile apical segments in petri dishes containing the control medium and waited 48 h for gametes to be released under controlled conditions. After the gametes were released, we carefully removed the apical segments and added the algal extracts at predetermined concentrations.

The development of the zygotes and survival rate was systematically monitored during the 14-day experimental period using standardised photo documentation (see 2.3.1). To maintain optimal culture conditions and prevent accumulation of metabolites, we changed the medium after seven days of incubation. The environmental parameters remained consistent with those of the first screening experiment to ensure comparability. This methodological continuity allowed a direct comparison of the results in both experimental stages, while the second phase focused specifically on concentration optimization.

2.3.3. Comparative effects of microalgal (Desmodesmus sp.) and macroalgal (AlgatronCifo®) extracts on G. barbata and E. crinita development

Following the concentration optimization phase (Section 2.3.2), we carried out comparative trials between the selected microalgal extract (*Desmodesmus* sp. at 0.07 mg mL^{-1}) and the commercial macroalgal biostimulant AlgatronCifo® (4.5 mL L^{-1}, Cifo S. p.A.), with Von Stosch (VS) serving as a control. The macroalgal product was selected due to its documented efficacy in enhancing fertility of adult *G. barbata* (Kaleb et al., 2023) and promoting seedling development of *Ericaria amentacea* (Malfatti et al., 2023). Parallel experiments with *G. barbata* and *E. crinita* allowed a direct comparison of the species-specific responses to these different biostimulant sources. The nutrient concentrations of AlgatronCifo® and VS medium are shown in Table 2, as reported in Kaleb et al. (2023) and Malfatti et al. (2023).

The experimental setup was standardised for both species (Fig. 3). For each treatment, we prepared three 1-L aquaria with twelve clay tiles with a diameter of 6 cm as substrate. Each tile received five fertile apical segments and was allowed to release gametes for four days before the biostimulant was applied. Seedling development and survival rate was photographically monitored (see 2.3.1), with *G. barbata* observed for 16

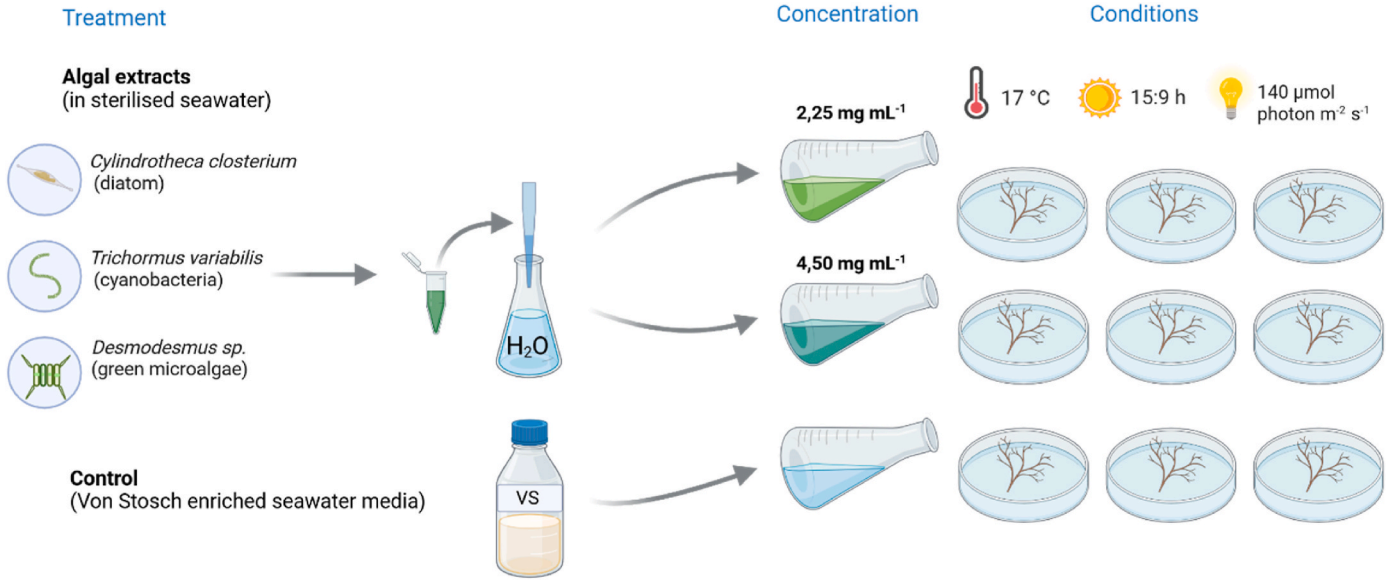

Fig. 1. Experimental setup for the preliminary screening of algal extracts on *Gongolaria barbata* germlings. Three microalgal extracts were tested at two concentrations (2.25 and 4.50 mg mL^{-1}), alongside a VS medium control. Each treatment was replicated in three 40 mL Petri dishes, with five fertile apices placed in each dish.

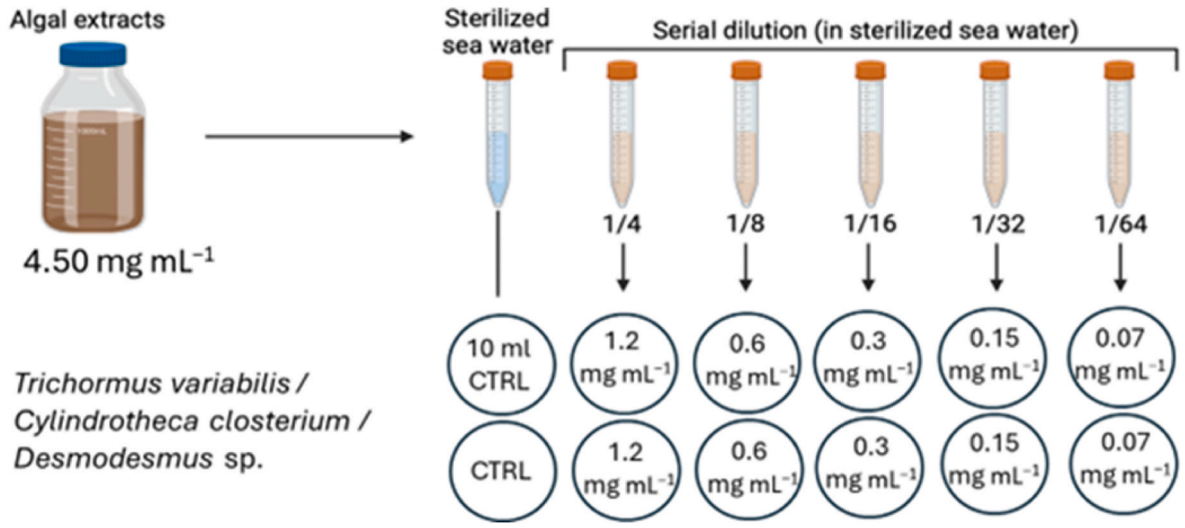

Fig. 2. Concentration optimization of microalgal extracts on *Gongolaria barbata* development. Experimental setup to test dose-dependent effects using duplicate 10 mL Petri dishes per treatment (three fertile apices each) with UVC-sterilized seawater control. Evaluated serial dilutions (0.07–1.2 mg mL^{-1}) of selected extracts identified in preliminary screening.

Table 2
Nutrients values of AlgatronCifo® and Von Stosch (VS) were reported by Kaleb et al. (2023) and Malfatti et al. (2023).

µg/L	Algatron pure product	Algatron at 4.5 mL L^{-1}	VS
TN	87466.70	530.60	
Urea-NO_2	6.10	0.03	
N-NO_2		1.30	1.27
N-NH_4	81546.67	373.50	0.75
N-NO_3	167.80	53.30	52.52
TP	200.10	7.03	
PO_4	214.70	1.97	
P-PO_4		1.17	5.3
N:P		324.58	10.29

days and *E. crinita* for 20 days to account for developmental differences. All cultures were maintained at 17 °C under a 15:9 light:dark cycle with an irradiance of 125–140 µmol photons $m^{-2}s^{-1}$, with media changes and continuous aeration beginning on day 10 of each experiment.

2.4. Statistical analyses and image processing

For the initial assessment of treatment effects (see 3.1), statistical analyses were performed using GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed to assess differences between treatments. Significance thresholds were set as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; and **** = $p < 0.0001$. Results are presented as mean

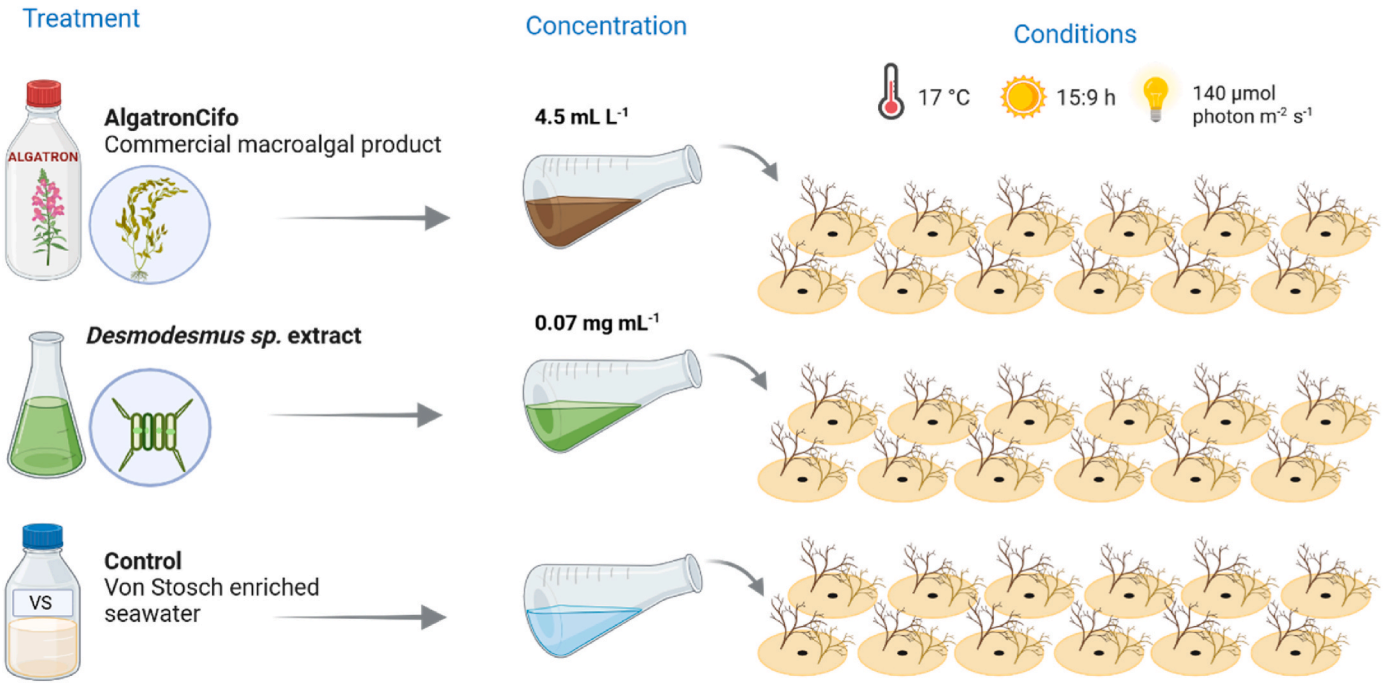

Fig. 3. Experimental setup for testing microalgal vs. macroalgal algal extracts on *Gongolaria barbata* and *Ericaria crinita* germlings development. Treatments included *Desmodesmus* sp. extract (0.07 mg mL^{-1}), the commercial macroalgal product AlgatronCifo® (4.5 mL L^{-1}), and a VS medium control. Each treatment was replicated in three 1 L aquaria, each containing twelve clay tiles (6 cm diameter).

± standard deviation (SD), with SD calculated and displayed for each condition.

Digital image analysis was performed using ACDSee software to process photographs of ex situ cultures of *Cystoseira s.l.* maintained at both the MBSP and CMRR facilities (see 3.2 and 3.3). For each image, we systematically assessed the success of germination within a defined area of 0.12 cm^2 by recording daily counts of live, dead, and deformed germlings. Mortality was determined by complete loss of pigmentation, indicating tissue decomposition, while deformed seedlings were classified as those showing partial pigment degradation or cell wall deformation without complete necrosis.

For a more comprehensive analysis of the culture data, we used R version 4.2.2 (R Core Team, 2020) in combination with PRIMER 7 software (version 7.0.24; PRIMER-e, 2017). A two-way ANOVA was implemented to investigate the effects of Treatment and Day (as fixed factors) on the percentage of live seedlings, including the assessment of interaction effects between these factors. Post-hoc tests were conducted using Tukey's Honest Significant Difference (HSD) test for pairwise comparisons between treatments and across time points. The WRS2 and AICcmodavg packages in the R environment were used for these analyses (see 3.2 and 3.3).

To further evaluate the effects of algal extract treatments and time on the response of *G. barbata* and *E. crinita* seedlings, a permutational multivariate analysis of variance (PERMANOVA) was performed in PRIMER. The analysis was based on a Euclidean distance similarity matrix, with Treatment and Day as fixed factors. The PERMANOVA used Type III (partial) sums of squares and was performed with 1000 permutations of the residuals under a reduced model to determine statistical significance (see 3.2 and 3.3).

3. Results

3.1. Characterization of the microalgae extracts

The chemical analysis revealed significant differences in the nutrient composition of the three microalgal extracts. Specifically, the green alga *Desmodesmus* sp. had the highest nitrogen content (4.5 mg/g DW), while *Cylindrotheca closterium* had the lowest value (1.9 mg/g DW) and the cyanobacterium *Trichormus variabilis* had an intermediate value (2.3 mg/g DW). The phosphorus content followed a similar pattern, with *Desmodesmus* sp. having 0.74 mg/g DW and both *C. closterium* and *T. variabilis* having lower and comparable values (0.311 and 0.34 mg/g DW, respectively) (Fig. 4).

Analysis of the phenolic content showed no statistically significant differences between the extracts, though notable variations were observed. *C. closterium* contained the highest phenolic values (209.44 mg GAE/g DW), followed by *Desmodesmus* sp. (157.33 mg GAE/g DW), while *T. variabilis* had the lowest values (125 mg GAE/g DW) (Fig. 5).

3.2. Concentration-dependent effects on G. barbata germlings

The initial screening (see 2.3.1) at higher concentrations (2.25 and 4.50 mg mL^{-1}) showed pronounced cytotoxic effects for all extracts. By day three of observation, all treatments showed abnormal germling development characterized by morphological deformities and inhibited growth, accompanied by proliferation of motile microorganisms. In contrast, the control cultures maintained in VS medium exhibited normal development without microbial contamination (see Supplementary Fig. S1). These negative effects prompted termination of the high-concentration experimental series.

Subsequent tests with optimized concentrations (see 2.3.2) showed extract-specific responses in *G. barbata* germlings (Figs. 6–7). Treatments with *T. variabilis* extracts consistently yielded the lowest survival rates at all concentrations tested. In contrast, *Desmodesmus* sp. at a concentration of 0.07 mg mL^{-1} showed the most favorable performance among the algal extracts, achieving survival rates exceeded only by the VS controls and promoting maximum germling length (604.18 μm).

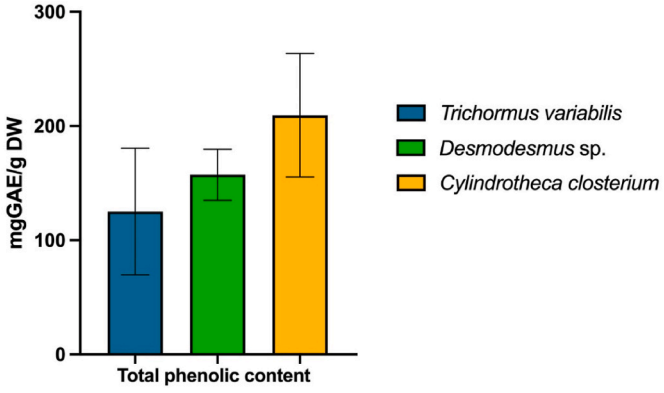

Fig. 5. Total phenolic content of the extracts of *Trichormus variabilis*, *Desmodesmus* sp. and *Cylindrotheca closterium,* expressed as mg gallic acid equivalents (mg GAE/g DW). A one-way ANOVA was used. Values are expressed as mean ± standard deviation (n = 3).

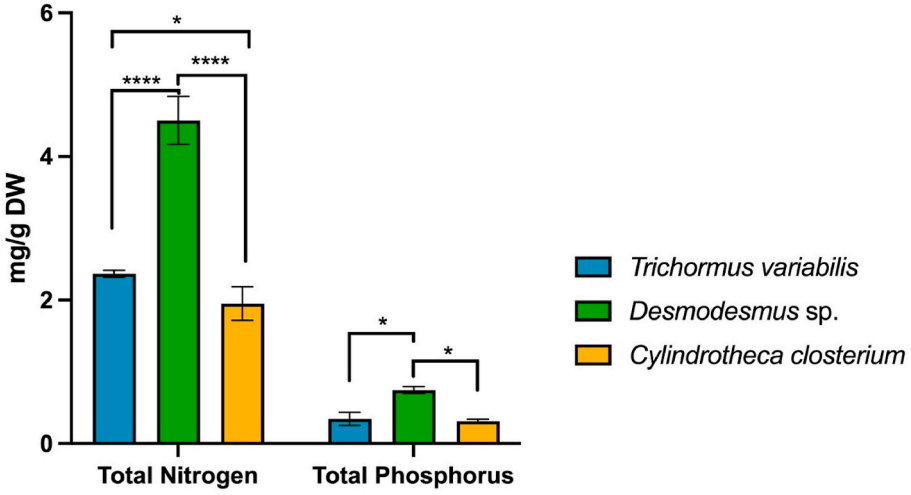

Fig. 4. Total nitrogen and phosphorus content in *Trichormus variabilis*, *Desmodesmus* sp., and *Cylindrotheca closterium* extracts. Values are expressed as mg per g of Dry Weight (mg/g DW). One-way ANOVA was used with * = $p < 0.05$ and **** = $p < 0.0001$. Values are given as mean ± standard deviation ($n = 3$).

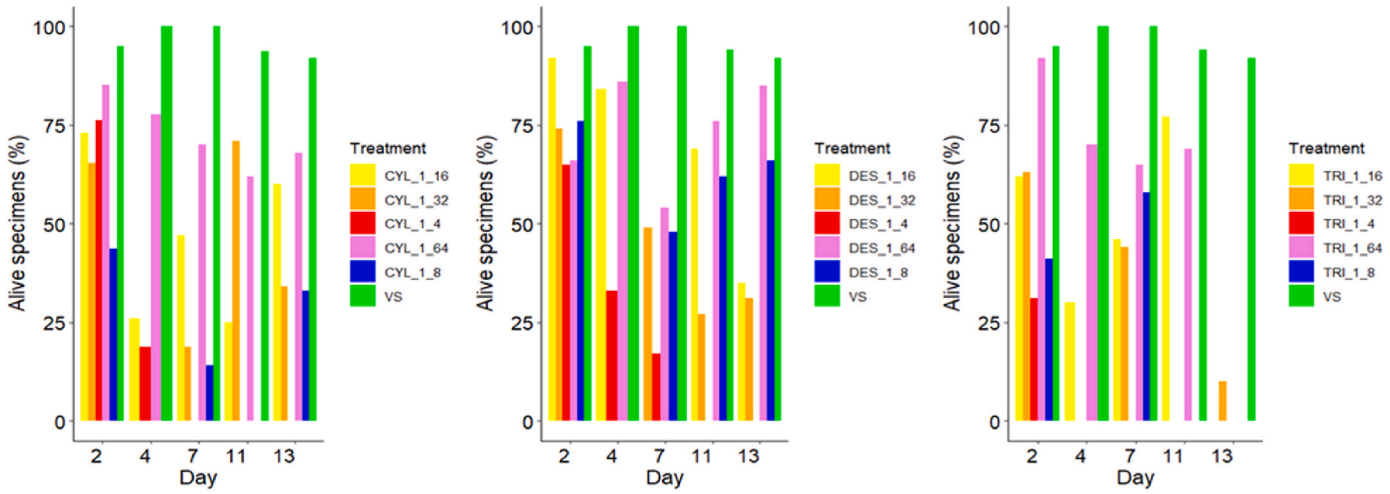

Fig. 6. Percentage of surviving *G. barbata* germlings in serial dilutions of the three algal extracts (see 2.3.2). The number of living germlings was counted in randomly photographed areas of 0.12 cm^2. CYL= *Cylindrotheca closterium*; DES = *Desmodesmus* sp.; TRI = *Trichormus variabilis;* VS =Von Stosch enriched seawater. Dilutions: 1/4 = 1.2 mg mL^{-1}, 1/16 = 0.3 mg mL^{-1}, 1/32 = 0.15 mg mL^{-1}, 1/64 = 0.07 mg mL^{-1}.

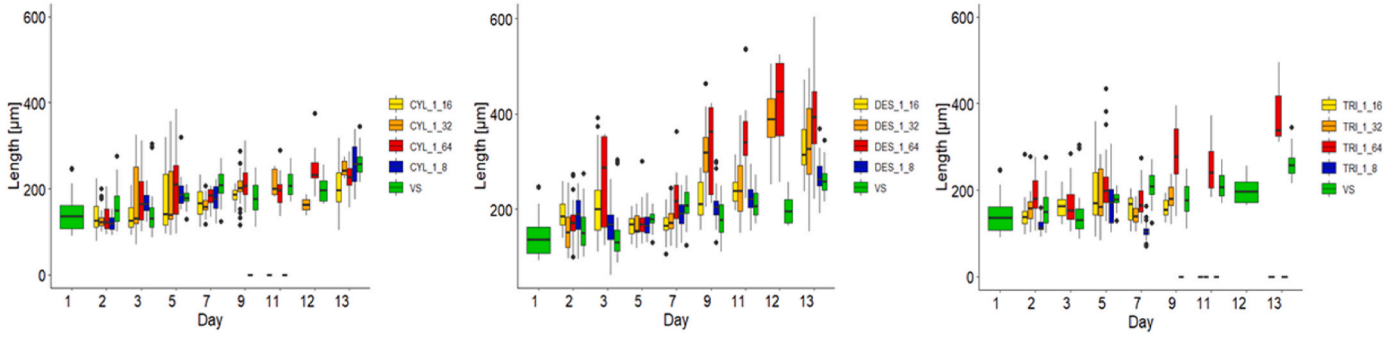

Fig. 7. Growth of *G. barbata* germlings (length in µm) in serial dilutions of the three extracts (see 2.3.2). Length measurements were made on living germlings in randomly photographed areas of 0.12 cm^2. The boxplots show the median, the first and third quartiles (boxes), the largest value no further than 1.5 × interquartile distance from the third quartile (upper whisker), the smallest value at most 1.5 × interquartile distance below the first quartile (lower whisker), outliers (dots). CYL= *Cylindrotheca closterium*; DES = *Desmodesmus* sp.; TRI = *Trichormus variabilis;* VS = Von Stosch enriched seawater. Dilutions: 1/4 = 1.2 mg mL^{-1}, 1/16 = 0.3 mg mL^{-1}, 1/32 = 0.15 mg mL^{-1}, 1/64 = 0.07 mg mL^{-1}.

The ANOVA results showed highly significant main effects on germling survival for both Treatment ($F = 124.25$, $p < 0.001$) and Day ($F = 123.97$, $p < 0.001$). Remarkably, the Treatment × Day interaction was not significant ($F = 14.64$, $p = 0.205$), suggesting that the effects of treatment on survival remained consistent throughout the trial. Post-hoc comparisons showed specific significant differences: the VS control outperformed both CYL_1_16 ($p < 0.05$) and CYL_1_32 ($p < 0.05$) treatments, while additional significant contrasts occurred between CYL_1_64 and CYL_1_4 ($p < 0.05$), and DES_1_64 versus CYL_1_8 ($p < 0.05$).

When measuring seedling length, the PERMANOVA results showed even more pronounced effects. Day accounted for most of the variation (59.4 %, $F = 195.13$, $p = 0.001$), while Treatment explained a smaller but still significant proportion (9.3 %, $F = 10.17$, $p = 0.001$). Importantly, we found a significant Treatment × Day interaction ($F = 5.34$, $p = 0.003$), indicating that the magnitude and direction of the effects of treatment on growth changed over time.

3.3. Species-specific responses to optimized biostimulant treatments

In *G. barbata* trials (see 2.3.3), survival rates after 16 days of culture were comparable for all treatments, though VS and *Desmodesmus* sp. (0.07 mg mL^{-1}) showed slightly higher values than AlgatronCifo® (Fig. 8). Growth patterns showed more marked differences, with VS

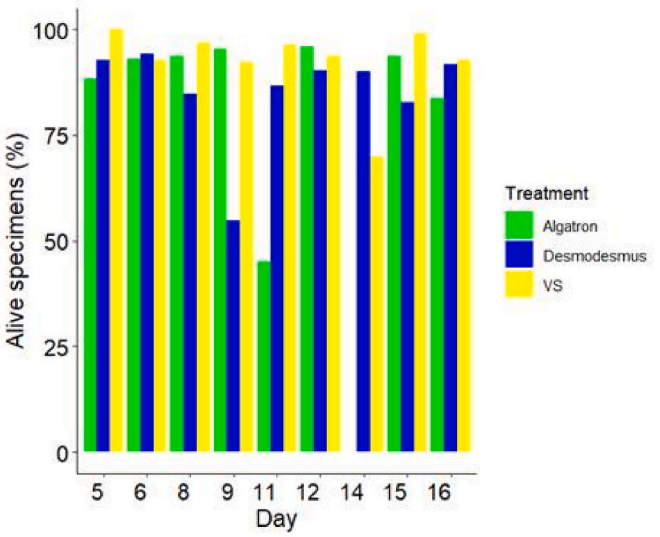

Fig. 8. Percentage of surviving *G. barbata* germlings after 16 days of cultivation in three treatments: *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and VS= Von Stosch enriched seawater (see 2.3.3).

control cultures reaching 983.95 μm, significantly higher than *Desmodesmus* sp. (395.26 μm) and AlgatronCifo® (339.4 μm) (Fig. 9).

Statistical models confirmed the absence of treatment effects on survival (ANOVA, F = 1.512, p = 0.250), while growth variation was significantly explained by both treatment (16.23 % of variation, PERMANOVA, F = 254.62, p = 0.001) and temporal factors (37.79 %). The significant Treatment × Day interaction (PERMANOVA, F = 44.55, p = 0.003) indicates dynamic growth responses over time. Pairwise tests confirmed statistically significant differences between treatments (see Supplementary Table S1).

Parallel experiments with *E. crinita* showed contrasting response patterns (Figs. 10 and 11). After 20 days of ex situ cultivation on clay tiles, survival rates were generally comparable for all three treatments, though subtle differences were apparent. The treatments containing *Desmodesmus* sp. extract (0.07 mg mL^{-1}) and AlgatronCifo® (4.5 mL L^{-1}) showed slightly higher survival rates compared to the VS control, as illustrated in Fig. 10.

The growth measurements showed clearer treatment effects. Treatment with *Desmodesmus* sp. extract resulted in a maximum seedling length of 773 μm on day 18 of cultivation. In comparison, the VS control reached a maximum length of 710 μm on day 20, while the AlgatronCifo® treatment showed more limited growth, reaching a maximum of 593 μm at the end of the experiment (Fig. 11). Despite these differences in final size, all treatments showed broadly similar growth trajectories throughout the duration of the experiment, as can be seen in Figs. 11 and 12.

The statistical analysis of the survival data yielded significant results. Treatment had a significant effect on survival percentage (ANOVA, F = 6.000, p = 0.007), while Day showed no significant effect (ANOVA, F = 1.641, p = 0.1367). Post-hoc Tukey HSD tests revealed specific significant differences between VS and the two treatments with AlgatronCifo® (p = 0.008) and Desmodesmus sp. extract (p = 0.037).

For seedling length measurements, PERMANOVA results showed highly significant effects. Day explained most of the variation (83.4 %, F = 448.59, p = 0.001), while Treatment explained a smaller but still significant proportion of the variation (0.21 %, F = 7.92, p = 0.001). The analysis also revealed a significant Treatment × Day interaction (F = 6.34, p = 0.001), which accounted for an additional 2.36 % of the variation. This interaction effect suggests that the influence of treatments on seedling length changed systematically over the course of the experiment. These patterns were confirmed by pairwise comparisons between treatments. The detailed results can be found in Supplementary Table S2.

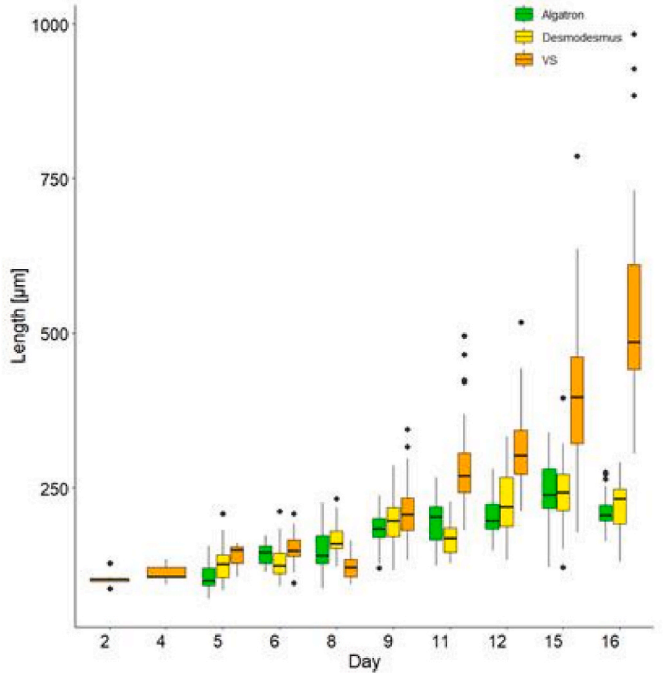

Fig. 9. Growth of *G. barbata* seedlings (length in μm) after 16 days of cultivation in *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and VS= Von Stosch enriched seawater (VS, control) (see 2.3.3). The boxplots show the median, the first and third quartiles (boxes), the largest value that is no further than 1.5 times the interquartile range from the third quartile (upper whisker), the smallest value that is no more than 1.5 times the interquartile range below the first quartile (lower whisker), outliers (dots).

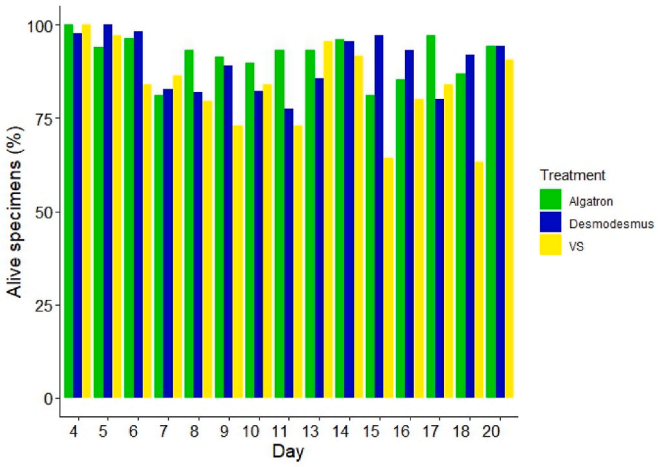

Fig. 10. Percentage of surviving E. *crinita* germlings after 20 days of cultivation in three treatments: *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and von Stosch enriched seawater (VS, control) (see 2.3.3).

4. Discussion

This study provides evidence that algal-derived biostimulants can significantly affect the early development of *G. barbata* and *C. crinita*, though their effects are highly dependent on the composition, concentration and target species of the extract. Our findings reveal several critical patterns that further the understanding of the application of biostimulants in macroalgal restoration.

The pronounced negative effects observed at higher extract concentrations (2.25–4.50 mg mL^{-1}) are consistent with previous reports of organic overload disrupting microbial balance in culture systems (Godlewska et al., 2016). The microbial bloom that accompanied these treatments likely created unfavorable conditions for germling development through multiple mechanisms: (1) competition for space and resources, (2) altered water chemistry via microbial respiration, and (3) potential proliferation of pathogens. This phenomenon illustrates the delicate balance required in ex situ culture systems, where organic input must be carefully calibrated to avoid destabilising the microbiome.

The superior performance of the *Desmodesmus* sp. extract at lower concentrations (especially 0.07 mg mL^{-1}) can be attributed to its distinct biochemical profile. With almost twice the nitrogen and phosphorus content of other extracts tested, this green algal extract likely delivers key nutrients in a more bioavailable form than conventional media. The organic nitrogen compounds in microalgal extracts could be particularly beneficial as they bypass the energy-intensive nitrate reduction pathway required for the assimilation of inorganic nitrogen in marine macrophytes. This metabolic shortcut could explain the initial growth advantage observed in *Desmodesmus*-treated seedlings compared to VS controls.

At lower concentrations, *Desmodesmus* sp. extract showed the highest biostimulatory effect on *G. barbata*, achieving growth and survival rates comparable to or even exceeding those of the control medium (VS). This effect is probably due to its high nitrogen and phosphorus content,

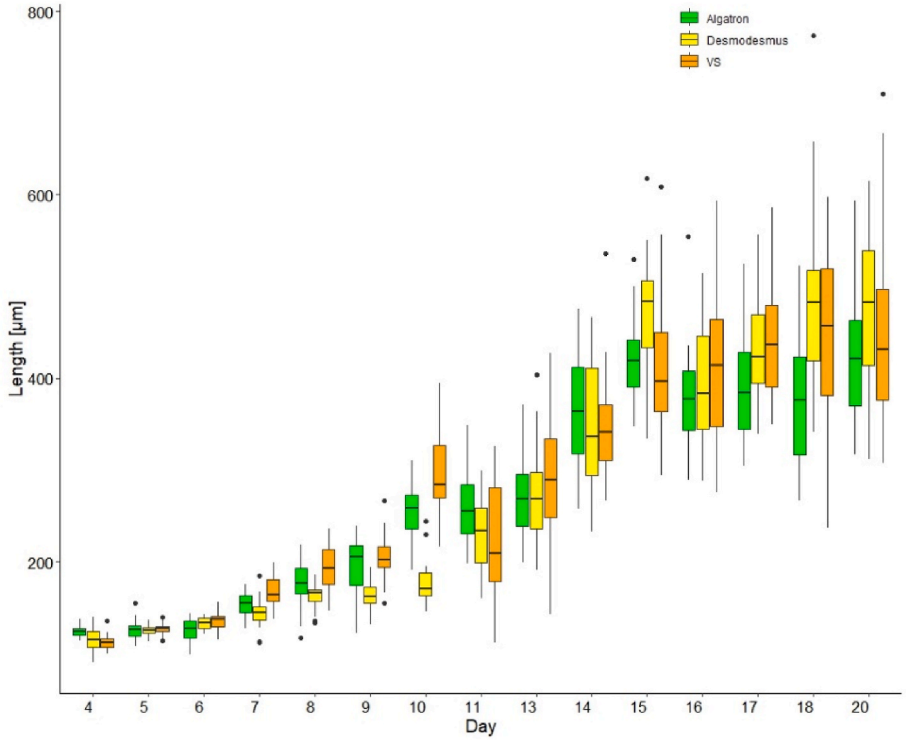

Fig. 11. Growth of *E. crinita* germlings (length in µm) after 20 days of cultivation in *Desmodesmus* sp. extract (0.07 mg mL^{-1}), AlgatronCifo® (4.5 mL L^{-1}) and Stosch's enriched seawater (VS, control) (see 2.3.3). The boxplots show the median, the first and third quartiles (boxes), the largest value that is no further than 1.5 times the interquartile range from the third quartile (upper whisker), the smallest value that is no more than 1.5 times the interquartile range below the first quartile (lower whisker), outliers (dots).

which is almost twice that of *Cylindrotheca closterium*, increasing its fertilizing capacity. In contrast, *Trichormus variabilis* performed consistently worse, possibly due to inhibitory secondary metabolites. The different performance of *C. closterium* could be related to its phenolic content, which is potentially beneficial at low concentrations but can be inhibitory at higher concentrations (Godlewska et al., 2016).

The comparative analysis between the two target species (i.e. *G. barbata* and *E. crinita*) revealed both shared trends and species-specific responses. In the 16-day cultivation of *G. barbata*, germling survival was high (~80–90 %) in all treatments. However, the VS medium yielded the longest thalli (~984 µm), and neither the extract of *Desmodesmus* sp. (0.07 mg mL^{-1}) nor the commercial biostimulant AlgatronCifo® performed significantly better than the control. While both extracts initially showed a growth-stimulating effect, this advantage diminished over time. This is particularly noteworthy considering that previous studies have reported positive effects of AlgatronCifo® on other species from the order Fucales, including seedling *Ericaria amentacea* (Malfatti et al., 2023) and adult *G. barbata* (Kaleb et al., 2023). In our case, neither the kelp-derived AlgatronCifo® nor the extract of *Desmodesmus* sp. outperformed the VS medium for *G. barbata* germlings. In contrast, *E. crinita* showed a clearer positive response to treatment with biostimulants. After 20 days of culture, the survival rate was significantly higher for the *Desmodesmus* sp. and AlgatronCifo® treatments compared to the control (VS). The seedlings treated with *Desmodesmus* sp. also attained the largest size (~773 µm). Though the absolute differences were modest, they were statistically significant. At the end of the experiment, the survival rate had decreased more in the VS and was about 10–15 % higher in the treated groups (see Fig. 10). These results suggest that algal extracts are particularly effective in supporting the early development of *E. crinita*, likely by mitigating physiological stress and compensating for nutrient limitations in the culture medium.

Interestingly, the extract of *Desmodesmus* sp. showed a slight advantage over AlgatronCifo® in promoting the growth of *G. barbata*, although both extracts proved to be similarly effective on *E. crinita*. This suggests that AlgatronCifo derived from *Macrocystis pyrifera*, which is rich in complex polysaccharides and phytohormones, may not fully fulfil the physiological requirements of early-stage *G. barbata* germlings. In contrast, the simpler biochemical composition of *Desmodesmus* sp. extract appears to be more compatible with the metabolic needs of the young thalli of both species.

Overall, these results underline the need for species-specific optimization in the application of algal biostimulants in macroalgal culture and restoration protocols. While both *G. barbata* and *E. crinita* responded positively to algal extracts, the magnitude and type of response differed, demonstrating that even closely related taxa can exhibit different physiological sensitivities to the same treatment.

These different results can be attributed to diverse physiological and molecular mechanisms by which algal extracts exert their effects. One of the most important mechanisms of action is the improved availability of nutrients. The extracts tested, especially that of *Desmodesmus* sp. (see section 2.1.4), contained significant amounts of organic nitrogen and phosphorus. These organic forms are more readily assimilated than their inorganic counterparts, bypassing enzymatic conversion steps and directly supporting important metabolic functions such as protein synthesis and cell division. Comparable fertilizing effects of microalgae biostimulants have been widely documented in agricultural systems (Chabili et al., 2024).

Additionally, algal extracts often contain essential micronutrients (e.g. Fe, Zn, Mn, Cu) and vitamins that act as cofactors in critical metabolic pathways (Godlewska et al., 2016). Brown algae in particular require exogenous vitamin B12 for their growth, which is usually supplied by symbiotic bacteria or enriched culture media (e.g. VS medium). Therefore, extracts from microalgae or seaweed enriched with B vitamins can support the metabolism of macroalgae more effectively than synthetic media alone (Godlewska et al., 2016).

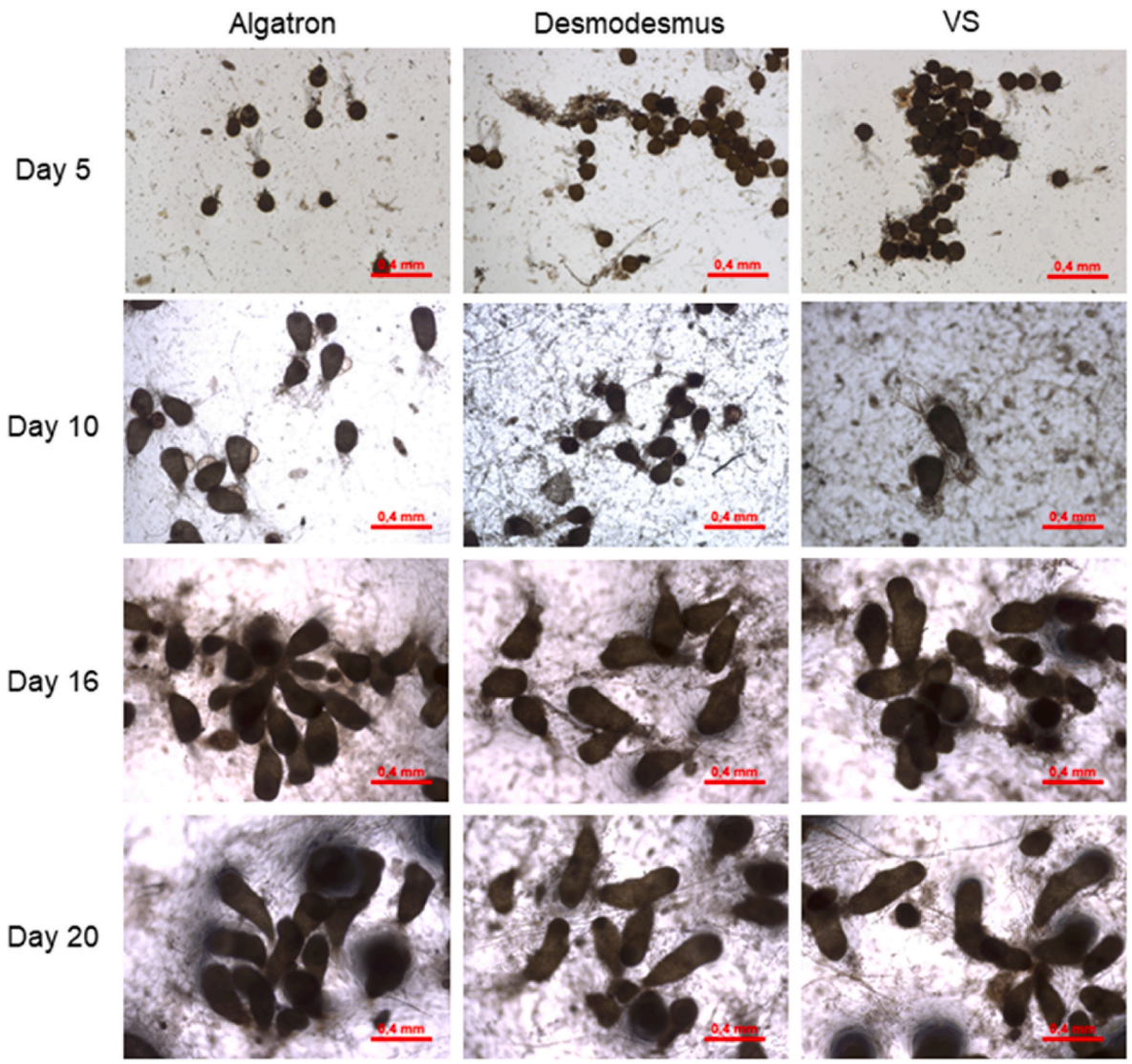

Fig. 12. Status of *Ericaria crinita* germlings under three treatments on Day 5, Day 10, Day 16 and Day 20 of the experiment in Rovinj.

Beyond their contribution to nutrition, algal biostimulants can provide phytohormone-like compounds, including auxins, cytokinins, gibberellins and abscisic acid. While these compounds are widely known for their regulatory role in terrestrial plant growth and stress responses, their functional importance is also increasingly recognized in macroalgal systems (Godlewska et al., 2016). Several studies suggest that the growth-promoting effect of algal extracts is not solely due to their mineral content, but that the presence of signalling molecules plays a crucial role (Ghaderiardakani et al., 2019).

In our study, the use of *Desmodesmus* sp. and *Macrocystis* (AlgatronCifo®) extracts probably led to such hormonal signalling. For example, cytokinins contained in green algal extracts are known to stimulate cell division and expansion in higher plants (Mazepa et al., 2021) and could similarly promote mitotic activity and thallus elongation in macroalgal germlings. Likewise, auxin-like compounds could influence the polarity and differentiation of zygotes — processes that are well documented in fucoid algae (Falace et al., 2018). While brown algae have endogenous hormonal pathways, the application of exogenous auxin analogues could enhance or modulate these mechanisms, potentially promoting rhizoid development and early thallus growth.

In addition, the presence of sulfated polysaccharides in AlgatronCifo®, such as laminarin, alginate and fucose-rich compounds, could influence cell wall architecture (Beuder and Braybrook, 2023) and signal transduction (Chi et al., 2018; Zhang et al., 2020). Although the exact hormonal composition of such extracts is still largely uncharacterised, these polysaccharides could interact with developmental signalling pathways.

In terrestrial systems, cytokinin-enriched extracts of *Desmodesmus* sp. have been shown to increase chlorophyll content and biomass by about 30 % (Mazepa et al., 2021), further supporting the potential of these compounds to promote macroalgal growth.

Another relevant mechanism is the stimulation of photosynthesis and overall metabolic activity. Algal biostimulants can increase pigment concentration and improve photosynthetic efficiency (Ali et al., 2022; Blunden et al., 1996; Yao et al., 2020), which ultimately increases energy production and carbon sequestration. In our experiments, such effects may have contributed to the greater growth and survival observed in the treated germlings. As chlorophyll biosynthesis depends on key nutrients such as nitrogen, magnesium and iron — all of which are commonly found in algal extracts (Godlewska et al., 2016) — these treatments likely supported the improved photophysiological performance.

The stress-protective properties of algal extracts deserve particular attention in restoration contexts. Early developmental stages of macroalgae are particularly vulnerable to abiotic stressors (e.g. temperature fluctuations, light intensity, osmotic shifts) and biotic pressures (e.g.

microbial colonisation, herbivory) (Ladah and Zertuche-González, 2007; Edwards, 2022). The *Desmodesmus* sp. extract contained increased amounts of polyphenols and antioxidant compounds, which may have helped to protect germling cells from oxidative damage. Although these effects do not directly stimulate growth, they contribute to improved overall viability.

Another aspect is the antimicrobial potential of algal biostimulants. Many algae and microalgae extracts have antimicrobial properties and have been shown to inhibit harmful microorganisms (Godlewska et al., 2016). The modest survival advantage observed in biostimulant-treated *E. crinita* seedlings could be due to reduced microbial pressure or selective promotion of beneficial microbial taxa. Malfatti et al. (2023) showed that kelp extracts altered the microbiome of *Ericaria amentacea* seedlings in a way that correlated with improved growth. Although we did not characterise the microbial assemblages in this study, it is plausible that similar probiotic effects occurred. Conversely, the application of high extract concentrations in the first experiment triggered a microbial bloom that overwhelmed the germlings, emphasising the critical importance of appropriate dosing.

The temporal dynamics of the responses to the treatments show that the positive effects of the algal extracts are particularly pronounced at certain stages of development. This pattern is especially evident during the crucial early post-settlement phase when the germlings undergo polarity and perform the first cell division. Our data show that the efficacy of treatment varies significantly over time, suggesting that the timing of application should be considered when designing the protocol as well as the composition of the extract. These results support the potential benefit of pulsed applications timed to coincide with critical developmental transitions, rather than continuous dosing, to maximise treatment efficacy while minimising potential adverse effects.

Within a restoration context, algal extracts represent a promising biotechnological tool to improve nursery performance. Their integration into growing protocols can improve vigour prior to outplanting while avoiding the ecological risks of direct nutrient enrichment (Eger et al., 2020; Earp et al., 2022; MacDonnell et al., 2022).

Taken together, these results strengthen the emerging view that algal extracts can significantly improve the fitness of early-stage macroalgal seedlings intended for restoration. The inclusion of *Desmodesmus* sp. extracts in rearing protocols could contribute to the development of more resilient seedlings for outplanting and ultimately improve restoration success.

Nonetheless, some limitations must be acknowledged. All experiments were conducted under controlled laboratory and nursery conditions. Field trials are essential to validate whether the benefits in the nursery phase also translate into better performance after outplanting under natural conditions. While Malfatti et al. (2023) reported that seedlings treated with biostimulants maintained their advantage post-transplantation, further validation across species, habitats and environmental conditions is essential.

Furthermore, our study focussed on early life stages; the responses of older seedlings or adults are still largely unexplored. Life stage–specific optimization may be necessary, as mature tissue may respond differently to nutrient or hormone supplementation (Kaleb et al., 2023). Future research should build on these findings along several key directions: (1) field validation of biostimulant-primed germlings in different environments and seasonal conditions; (2) transcriptomic or proteomic studies to elucidate mechanisms of action such as upregulation of nitrogen assimilation genes or stress-related genes; (3) detailed assessment of biostimulant-induced microbiome shifts and their functional consequences; and (4) broader exploration of biostimulant applications in benthic macroalgal restoration.

As marine ecosystems are exposed to increasing degradation, biotechnological tools orientated towards ecological processes are urgently needed. Algal extracts represent a scalable, cost-effective and ecologically sound strategy by utilising natural metabolic and signalling compounds. With further refinement, this approach can increase the success of restoration efforts not only for *Cystoseira* s.l. forests, but also for other important coastal habitats such as kelp forests and seagrass beds.

CRediT authorship contribution statement

Ana Lokovšek: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Martina Orlando-Bonaca:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Edi Gljušćić:** Writing – review & editing, Methodology, Investigation, Data curation. **Andrea Bilajac:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ljiljana Iveša:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Alberta Di Cave:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Saverio Savio:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Federico Ortenzi:** Writing – review & editing, Methodology, Investigation. **Domen Trkov:** Writing – review & editing, Investigation, Conceptualization. **Roberta Congestri:** Writing – review & editing, Methodology, Conceptualization. **Annalisa Falace:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Data availability

Most data presented in this study are contained within the article and Supplementary Materials (Tables S1 and S2). The rest are available on request from the corresponding author.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Chatgpt 4.0 in order to improve language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107411.

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