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Ex-situ restoration of the Mediterranean forest-forming macroalga *Ericaria amentacea*: Optimizing growth in culture may not be the key to growth in the field

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ABSTRACT

Evidence of local and regional declines in the canopy-forming alga *Ericaria amentacea*, a foundation species of diverse marine forest communities on exposed Mediterranean coasts, have spurred restoration efforts focused on sustainable *ex-situ* techniques. The need to balance the costs of culture maintenance and the susceptibility of early life stages to stressors in the native habitat, including rapid, often extreme shifts in temperature, hydrodynamics and nutrient availability, have driven current efforts to create a culture environment that primes seedlings for outplant, increasing their resilience rather than maximizing growth. We tested the effects of 1) higher culture temperature (25 °C) combined with wave simulation and 2) reduced nutrient loads (10% of standard protocol) with wave simulation on post-culture and post-outplant outcomes relative to optimal growth conditions in established protocols (20 °C, no waves, high-nutrient culture medium). While increased temperature and water motion negatively affected seedling growth in culture, and higher nutrients caused oxidative stress likely associated with enhanced epiphyte overgrowth, these effects were not clearly translated into patterns of long-term growth in the field. Instead, survival in the initial days post-outplant appeared to be the bottleneck for restoration potential, where substrates with persisting seedlings at one month were generally found with flourishing juveniles at four months. Larger clumps of seedlings, in turn, were strongly associated with both initial survival and future growth. These results underscore the importance of the zygote settlement phase to establish high seedling densities, which may be optimized by phenological monitoring of the donor population. They also suggest that less-controlled, more environmentally-realistic culture conditions involving the introduction of mild stress may enhance the survival of early life stages of *E. amentacea* during the transition to the native environment, providing a means to simultaneously reduce human resource costs in culture and move toward scaling up.

1. Introduction

Marine forests provide habitat, refuge and food for diverse associated assemblages (Wernberg and Filbee-Dexter, 2019; Eger et al., 2023), but are undergoing range reductions and loss (Eger et al., 2022; UNEP, 2023) due to varied human stressors, including eutrophication, habitat destruction, warming and extreme meteorological events associated with climate change (Airoldi and Beck, 2007; Gorman and Connell, 2009; Smale, 2020). These canopy-forming species are replaced by less sensitive species, often algal turfs (Filbee-Dexter and Wernberg, 2018; Pessarrodona et al., 2021) whose reduced biomass (Smale, 2020), habitat structure (Pessarrodona et al., 2021) and potentially lower rates of productivity (Krumhansl et al., 2016; De La Fuente et al., 2019a; Pessarrodona et al., 2022) result in loss of important ecosystem services.

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Moreover, once established, positive feedbacks within turfs may prevent the re-establishment of marine forest species, even if stressors are removed (Airoldi, 1998; O'Brien and Scheibling, 2018; Layton et al., 2019).

In the Mediterranean, marine forests are primarily composed by species of the genus *Cystoseira sensu lato*. In the early 2000s, evidence of extensive range reductions and local *Cystoseira s.l.* species loss (Thibaut et al., 2005, 2015) spurred discussion and preliminary restoration efforts (Falace et al., 2006; Susini et al., 2007; Gianni et al., 2013). More recently, the need for species restoration has been recognized at both the European (Biodiversity Strategy for 2030: EC, 2020) and global level (UN Decade of Ecosystem Restoration: UN, 2019), with the funding of initiatives to establish standardized protocols for *Cystoseira s.l.* restoration that move toward scaling up (e.g. MERCES, 2016–2020; ROC-POP Life, 2018–2021; AFRIMED, 2019–2022; REEForest, 2022–2026; MARES-NBFC, 2022–2025; MER-ISPRA, 2022–2026). In contrast to early efforts involving transplantation, these initiatives focus on development of sustainable, *ex-situ* methods to culture seedlings in the laboratory for outplant into the field without over-exploitation of donor sites (Falace et al., 2018; De La Fuente et al., 2019b; Cebrian et al., 2021; Clausing et al., 2022; Smith et al., 2023).

Ericaria amentacea, a foundation of diverse communities on exposed, rocky Mediterranean shores, is used as a bioindicator for ecological quality of midlittoral coasts (CARLIT: cartography of littoral and upper-sublittoral rocky-shore communities; Ballesteros et al., 2007; Mangialajo et al., 2007; Blanfuné et al., 2017) due to its sensitivity to the human impacts to which it may be particularly exposed in its native vertical zone at the interface between land and sea. The naturally high environmental stress of the habitat of *E. amentacea*, however, complicates restoration efforts, particularly *ex-situ* methods with vulnerable early life stages which experience high post-outplant mortality (De La Fuente et al., 2019b; Clausing et al., 2022), representing the first bottleneck to restoration potential. Consequently, after initial work by the ROC-POP Life group to establish protocols for culture (Falace et al., 2018) and implantation at sea (De La Fuente et al., 2019b), efforts have focused on the fine-tuning of culture conditions to promote seedling resilience in the field, rather than maximal growth in culture (hereafter optimal growth conditions) (Clausing et al., 2022). Culture under optimal growth conditions may not prepare these early life stages for the stress they will experience in the native environment, from extreme and fluctuating temperatures, UV exposure, flow and wave shock to low or intermittent nutrient availability (Davison and Pearson, 1996). Further, optimal growth culture conditions also highly favor epiphytic growth over extended culture periods (Orlando-Bonaca et al., 2021; Clausing et al., 2022; Lardi et al., 2022; Khaya et al., 2024). In our previous work, we showed that both survival and growth in the native habitat was strongly enhanced by exposure to mild, variable light and heat stress in culture (Clausing et al., 2022). Work on other *Cystoseira s.l.* species has also shown that mild physical disturbance in the form of water turbulence improved preparation of juveniles (3 months) for habitat conditions by inducing thalli thickening and holdfast development (*Gongolaria barbata*: Lokovšek et al., 2023; *G. nodicaulis*: Khaya et al., 2024).

In this context, we tested if exposure to mild physiological and mechanical stress in culture can prime seedlings for the transition to the native habitat by improving their resilience to environmental stress and thereby enhancing survival and growth in the initial post-outplant period (days to weeks). Modified culture conditions from established protocols were established from the first days post-settlement and involved 1) higher culture temperature combined with simulated wave action and 2) reduced nutrient loads (10% of standard protocol) and wave simulation.

We expected that seedlings cultured under higher temperatures would show more resilience to summertime extremes after outplanting in mid-July and that wave motion might promote higher allocation of resources to thalli structural resistance or rhizoid attachment, reducing detachment in the field and further promoting survival. We hypothesized that reduced, more environmentally-realistic nutrient loads would have secondary benefits to seedlings in culture by reducing epiphyte overgrowth, particularly in combination with wave simulation to (a) inhibit opportunistic species that favor stagnant water and (b) limit boundary layer effects, promoting gas and ion exchange on the algal surface.

2. Materials and methods

2.1. Study sites

E. amentacea, once dominant along the rocky coast of western Italy, has exhibited substantial declines throughout the region. In particular, in the easternmost part of the Ligurian Sea, we identified the Cinque Terre MPA as a stretch of coast in need of restoration of this species. Herbarium records show the historical presence of *E. amentacea* in this area (De La Fuente et al., 2018); disappearance in the early 1900s was likely due to water pollution and heavy sedimentation associated with excavation activities in the Magra River watershed (outflow ~25 km east). Water quality has since been restored to its natural oligotrophic state, with annual average levels of NO_3, NH_4, and PO_4 all below 1 μM (ARPAL data 2016–2022, Table S1). Further, direct human impacts have been removed with the establishment of the MPA in 1997. Yet, the rocky coast continues to be dominated by mixed coralline turfs and short (<12 cm frond length), fragmented stands of *Cystoseira compressa* (unpubl. quadrat data 2018–2019 and De La Fuente et al., 2018*)*; the continued absence of *E. amentacea* is likely linked to limited dispersal ability (Clayton, 1990; Johnson and Brawley, 1998). Outplanting took place in the most protected part of the MPA (Punta Mesco: 44° 8′2.95″N – 9°38′10.10″E), following characterization to verify suitability and compatibility with the donor site. The donor site consisted of a stretch of the Ligurian coast within the Portofino MPA (Punta Chiappa-Portofino: 44°19′21″N – 9°08′46″E) with a healthy population of adult *E. amentacea*. The site is relatively removed from large watersheds (Recco, Genova) and hosts a stable marine forest community (CARLIT data obtained with permission from ARPAL, the Ligurian Regional Agency for the Protection of the Environment). All activities within the MPAs were conducted with authorization (permit nos. 1/2020-prot 1716/2-1-1, 1/2021-prot 83/2-1-1, and 1692/2-1-1).

2.2. Culture establishment and experimental conditions

Fertile apices of *E. amentacea* were collected on June 22, 2020 and July 05, 2021 and transported to laboratory facilities at the University of Genova (<1 h) in cold, dark conditions where they were cleaned of epiphytes and sediment and rinsed with filtered seawater. After 24 h in the dark at 5 °C, apices were placed on round clay tiles (3 apices per 4.5 cm diameter tile, *sensu* Falace et al., 2018) in 20 °C UV-sterilized, filtered seawater and left overnight. When apices were removed the following morning, zygotes were observable on the tiles. After 24 h undisturbed to allow embryo attachment, tiles were randomly assigned to experimental aquaria containing fresh culture media with bubblers for oxygenation. Except where noted below (2.2.2) culture media was UV-treated Von Stosch's enriched filtered seawater (VS). Cultures were maintained in a temperature-controlled room set at 20 °C under a light:dark cycle of 14:10 h (LED aquaculture lamps: Maxspect RSX 150). Following Clausing et al. (2022), light intensity varied hourly around a constant mean (125 μmol photos m^{-2} s^{-1}, ranging 75–200). To control for variation in light intensity with position under the lamp, aquaria and tiles within aquaria were randomly repositioned every 3 days when culture media was refreshed. All treatments received additional antibiotics and GeO_2 to reduce growth of bacteria and diatoms when needed. Tiles were labelled with shellfish tags (Hallprint, Australia) and photographed individually at 3 time points during culture: during the first water change (day 3, at initiation of experimental treatments), mid culture (day 12) and before outplant (day 18).

Experiments in 2020 consisted of two parallel factorial experiments examining the interacting effects of 1) increased temperature (25 °C *vs.* 20 °C) and wave simulation (+/−) and 2) reduced nutrient content of the culture medium (N, P at 10%) and wave simulation (+/−) on seedling growth in culture and subsequent post-outplanting success. Each experiment had two levels of two crossed factors, giving $n = 4$ treatments. Based on results from 2020 and the goal of obtaining post-culture measures of oxidative stress (described below in 2.3), the nutrient treatments were repeated in 2021. No other factors were considered in order to maximize replication and ensure availability of tissue for biochemical analyses.

2.2.1. *Increased temperatures and wave simulation*

While previous work has shown optimal seedling development and growth in culture at 20 °C (Falace et al., 2018; De La Fuente et al., 2019b), we found that periodic warmer temperatures in culture (heat pulse: 25 °C one day in three) improved growth of outplanted seedlings in the sea (Clausing et al., 2022). Herein, we tested if sustained warmer temperatures in culture would have equivalent benefits on field fitness and if wave exposure from the initial recruit stages could improve resilience to detachment. Half of the seeded tiles ($n = 160$) were assigned to the control temperature treatment at 20°; the other half were maintained at 25 °C with electric resistors ($n = 4$ aquaria). Temperatures were continuously monitored with data loggers (HOBO®). Wave simulation, assigned randomly to half of the aquaria at each temperature ($n = 40$ tiles per treatment, 20 tiles per aquaria), was achieved using a wave pump (Jebao DW-5 Wavemaker, Netherlands). The wave mode and flow rate (500 L h^{-1}) were selected to best approximate water movement experienced by *E. amentacea* in the intertidal habitat, given that sea conditions vary between extremes with the weather. The unidirectional flow produced by the wave pump reflects and refracts between the opposing sides of the tanks, creating irregular currents and wave action. Seedling detachment was not noticeably enhanced by wave action.

2.2.2. *Reduced nutrients and wave simulation*

In the established culture procedure (Falace et al., 2018), seedlings of *E. amentacea* are cultivated in Von Stosch enriched medium (VS; von Stosch, 1964) containing 500 μM of NO_3 and 30 μM of PO_4. In contrast, the Ligurian coast is a generally oligotrophic environment (<1 μM NO_3 and PO_4), with surface waters becoming ultraoligotrophic during the summertime reproductive period (1.3 μM lower total N on average than in winter; Table S1). Moreover, in the intertidal range of *E. amentacea*, nutrient uptake is only possible when algae are inundated by wave action. We tested (a) if reduced nutrient levels in the culture media could sustain growth without inducing nutrient limitation or affecting field fitness and further benefit seedlings by reducing epiphyte overgrowth and (b) if water motion altered these effects. Seeded tiles were randomly distributed to aquaria containing filtered and ultraviolet-treated seawater enriched with 500 + 30 μM (i.e. standard VS) or 30 + 3 μM $NO_3 + PO_4$ (~10% VS; $n = 80$). Proportions of vitamins and antibiotic additions did not differ between treatments. Half of aquaria in each nutrient treatment were assigned to wave simulation, as above ($n = 40$ tiles per treatment, 20 per aquaria). All aquaria were maintained at 20 °C. The molar N:P ratios were selected to approximate values measured in the native habitat (mean annual N:P of 15 at Punta Mesco, Table S1) and to remain constant between treatments (VS: 16.7 and 10% VS: 10) to avoid the risk of inducing secondary nutrient limitation.

In 2021, the identical VS (500 + 30 μM N + P) and ~10% VS (30 + 3 μM N + P) nutrient treatments were randomly assigned to all tiles ($n = 118$). Aquaria were maintained at 20 °C without wave pumps and all other conditions were as described above. At the end of the culture period, seedlings from a proportion of tiles in both treatments were harvested for biochemical analyses as described in **2.3**.

2.3. *Biochemical parameters of oxidative stress and damage*

Oxidative stress was analyzed in the seedlings from both nutrient treatments (VS and reduced nutrients; $n = 5$ replicates). At the end of the culture period, entire seedlings were carefully collected with tweezers from a random selection of tiles and immediately frozen at −80 °C. Prior to collection, tiles were gently cleaned with a soft brush to remove epiphytes, and additional care was taken to avoid harvest of epiphytized tissue where possible. Sample extraction was performed following Luo and Liu (2011); ~60 mg of algal tissue was homogenized in 50 mM potassium phosphate buffer (with 0.25% triton and 1% polyvinylpyrrolidone, pH 7.0) in a proportion 1:5 w:v using TissueLyser II QIAGEN® set at a frequency of 30 s^{-1} for 90 s. After centrifugation of the homogenate at 12,000×*g* for 20 min at 4 °C, the supernatant was collected and stored at −80 °C. The total protein content of each sample was measured according to the Bradford (1976) method using bovine serum albumin as standard (0.1–0.5 mg mL^{-1}; $r^2 > 0.98$). Absorbance was read at 595 nm.

The content of reactive oxygen species (ROS) was assessed by absorbance at 485 nm λ_{exc} and 530 nm λ_{em} in dichlorofluorescein-diacetate (DCFH-DA, 10 mg mL^{-1} in DMSO) after 30 min incubation at 37 °C using an EnSight™ plate reader (PerkinElmer) (Deng et al., 2009). Data are expressed as Arbitrary Fluorescence Units (AFU) mg $prot^{-1}$.

Superoxide dismutase (SOD) activity was measured by adding samples to 50 mM potassium phosphate buffer with added 0.1 mM EDTA (pH 7.8), 50 μM hypoxanthine, 10 μM cytochrome C and bi-distilled water. After 3 min of incubation, xanthine oxidase (XOD 1.87 mU mL^{-1}) was added, and the reading was carried out at 550 nm in a 6715 UV/Vis spectrophotometer (Jenway, UK). The change in absorbance of samples was read for 1 min against the blank (homogenization buffer only). SOD activity was determined by measuring the degree of inhibition of cytochrome C reduction by the superoxide anion generated by the xanthine oxidase/hypoxanthine reaction (McCord and Fridovich, 1969). Activities are given in SOD units min^{-1} mg $prot^{-1}$, where 1 SOD unit = 50% inhibition of the xanthine oxidase reaction.

Catalase (CAT) activity was measured by the decrease in 240 -nm absorbance of a 10 mM H_2O_2 solution. The enzymatic activity is expressed as mmol min^{-1} mg $prot^{-1}$ (Greenwald, 1985).

Lipid peroxidation (LPO) was determined according to the protocol developed by Buege and Aust (1978). Homogenates were obtained by mechanically lysing tissues in a solution with 20% Trichloroacetic acid (TCA) in a proportion 1:6 w:v (30 s^{-1} for 90 s, TissueLyser II QIAGEN®). After centrifugation at 12,000×*g* for 20 min at 4 °C, the resulting supernatant was collected and incubated with 0.5% 2-thiobarbituric acid (TBA) and 20% TCA at 96 °C for 25 min and then transferred to ice to stop the reaction. The absorbance of the reaction was read at 532 nm in an EnSight™ plate reader (PerkinElmer, Milan, Italy). LPO levels are expressed as nmol of MDA formed g^{-1} ww.

Samples for all measures were analyzed in triplicate.

2.4. *Field outplanting and monitoring*

After a ~3 week culture period (2020: 20 days; 2021: 21 days), tiles were transported to Cinque Terre MPA for outplant. Tiles were fixed to the rock with screws fitted with neoprene washers to prevent tile erosion by the screw head. Tiles were surveyed for survival and photographed at 2, 4 and 17 weeks post-outplant as permitted by weather conditions and restrictions caused by the covid-19 pandemic. Survival was noted as presence of at least one individual on the tile, recording absence to differentiate from tiles lost or unable to be found.

While this paper focuses on culture treatment effects on the periods of transition and establishment in the native habitat, we discuss non-specific results of extended monitoring (1 and 3 years) on thallus height and fertility.

2.5. Photo and statistical analysis

2.5.1. Photo analysis

Algal percent cover at each time point during culture (3, 12, 18 days) and field monitoring (2, 4, 17 weeks) was analyzed by manual thresholding the images to select pixels in the colour spectrum corresponding to *E. amentacea* tissue (ImageJ v1.52, National Institute of Health, USA). We also assessed the level of epiphyte growth, if any, and the degree of spatial aggregation of seedlings (i.e. clumping) at the time of outplanting (18 d culture photos). Epiphytes were visually estimated into cover categories (negligible, low, medium and high). Clumping was evaluated by the number and size distribution of polygons resulting from aggregated individuals identified in the thresholding process (sensu Clausing et al., 2022).

2.5.2. Culture growth

To check for random differences in embryo settlement among treatments, we ran linear models on percent cover at the start of experimental treatments (3 days' culture, after the first change of medium; assumptions of parametric statistics satisfied). The cover of *E. amentacea* over the tile substrate was examined with linear mixed models with percent cover at 12 and 18 days' culture as the response variable and experimental treatments and culture day as fixed factors (package *glmmTMB*). Cover at day 3 was modelled as a covariate, and tile ID was included as a random effect. No transformation was needed as data met assumptions for a gaussian residual distribution (packages *DHARMa* and *performance*). Models were inspected and simplified using the Akaike information criterion (Zuur et al., 2009). Where interactions were significant, they were further compared using least-squares means (package *emmeans*: Lenth, 2024))

To assess the contribution of different clump sizes to algal cover among treatments, we calculated the proportion of total algal area comprised by 6 broad size classes of polygons (0–450 mm^2) obtained from thresholding (1: >0–2 mm^2, 2: >2–10 mm^2, 3: >10–30 mm^2, 4: >30–50 mm^2, 5: >50–100 mm^2, 6: >100 mm^2, hereafter depicted as [X, Y) for each bin); i.e. for a tile *j*, the contribution for a given size class $proportion = \frac{\sum_{i=1}^{n} X_i}{\sum_{k=1}^{N} X_k}$, where $x_{i \ldots n}$ are the areas of *n* polygons in a given size class, $x_{k \ldots N}$ represents the area of all polygons for tile *j*, and $\sum_1^6 proportion = 1$. Permutation-based multivariate analysis of variance (PERMANOVAs on bray dissimilarity matrices; $n = 9999$) assessed treatment differences in clump size contributions to cover (package vegan, function *adonis2*); following significant PERMANOVA (exp1 only), patterns were examined by ordination (non-metric multidimensional scaling), and univariate analyses were performed for each size class. Although data did not meet homogeneity of multivariate dispersions (PERMDISP, $F_{3,154} = 5.3$, $p = 0.002$), PERMANOVA is robust to violation of this assumption in the case of a balanced design (Anderson and Walsh, 2013).

2.5.3. Field growth

For both experiments, differences in percent cover between treatments at the time of outplant were compared with factorial ANOVA.

Changes in cover of *E. amentacea* (as proportion of tile substrate covered: continuous data bounded [0,1]) over 17 weeks post-field outplant were analyzed with generalized linear mixed models (package *GLMMTMB*). Fixed factors included time (i.e. monitoring events: 2, 4, and 17 weeks) and culture treatments: exp1 – temperature (20 °C vs 25 °C) and water motion (+/−); exp2 – nutrients (standard VS vs 10% VS culture medium) and water motion (+/−). Total cover of seedlings at the time of outplant (initial cover) and their distribution on the tile substrate (i.e. clumping measures) were modelled as covariates. Clumping measures included the maximum clump (polygon) size, the percent contribution of medium-large clumps to total area (polygons 10–50 mm^2), and the skewness of the polygon size distribution (unbinned data), which gives a measure of the relative prevalence of larger clumps. The inclusion of tile ID as a random effect accounted for repeated observations over time. Model inspection and simplification using the variance inflation factor (VIF <3: Zuur et al., 2010) and Akaike information criterion resulted in best-fit models fitted with beta distribution and logistic link (Douma and Weedon, 2019), including a zero-inflation factor to account for the prevalence of zeroes (independent of predictor variables, ~1; $p < 0.0001$). Diagnostic plots of scaled residuals confirmed that assumptions were met (packages *DHARMa* and *performance*). A posteriori comparisons of significant interactions were made by least-squares means (package *emmeans*).

Clumping at the time of outplant was further examined for its role in survival in the first weeks and months. Non-metric multidimensional scaling (nMDS; bray dissimilarity of binned data: 11 size categories determined from exploratory histograms) was used to visualize differences in survival, where tiles were grouped by the last date in which *E. amentacea* was observed present (2, 4 or 17 weeks) with all treatments from both experiments combined.

3. Results

3.1. Culture establishment

At the initiation of experimental treatments, embryo settlement was not significantly different among tiles assigned to each treatment in either experiment (3 days post gamete release; Table 1). Thus, subsequent differences in growth and cover among treatments can be attributed to treatment effects rather than underlying differences in zygote settlement or initial cell divisions. Careful seeding of every tile with three apical tips from a mix of individuals and evidence that zygotes generally sink and settle beneath the site of fertilization (Clayton, 1990; Falace et al., 2018) maximize the probability of even recruitment. Cover at day 3 was then used as a covariate in statistical models to account for artefacts of random differences in zygote settlement among tiles prior to application of treatments.

3.2. Increased temperatures and wave simulation

Culture: Relative to culture at 20 °C, higher temperature (25 °C) resulted in reduced seedling survival and growth as measured by cover, independent of wave motion, with effects beginning early in culture (medians of 3.31 *vs* 6.50% cover at 12 d; $t_{309,} = 3.07$, $p = 0.002$) and increasing over time to 3.76 *vs* 8.17% at the end of the culture period ($t_{309,} = 5.86$, $p < 0.0001$ at 18 d; post hoc comparisons following a significant interaction of temperature by days in culture; Table 2; Fig. 1). Wave motion similarly impeded the development of seedling cover in culture (interaction of wave motion by days in culture: Table 2), where tiles without wave motion (bubblers and aeration pumps only) had 1.5 and 2 times more cover than those with wave pumps after 12 d ($t_{309,} =$

Table 1

Differences in post-settlement cover among tiles at the onset of experimental treatments (linear models, assumptions checked and met).

	Estimate	SE	*t*-value	*p*-value
1: nutrients * wave motion				
intercept	5.916	0.505	11.709	≪0.001
nutrients	−0.556	0.710	−0.783	0.435
wave motion	0.215	0.710	0.303	0.763
nutrients: wave motion	−0.744	1.001	−0.743	0.459
			R^2	0.0069
2: wave motion * temperature				
intercept	5.916	0.503	11.764	≪0.001
wave motion	0.215	0.707	0.304	0.762
temperature	−1.187	0.707	−1.680	0.095
wave motion:temperature	−1.197	0.996	−1.201	0.232
			R^2	0.070

Table 2
Growth as percent cover of *Ericaria amentacea* germlings in culture (cover assessed at 3, 12 and 18 days) in treatments of optimal (20 °C) or increased (25 °C) temperature with or without wave motion. *Cover at day 3* was modelled as a covariate, while the other two dates form a fixed time factor *days culture* (GLMM, gaussian family of residual distributions; assumptions checked and met with packages DHARMa and performance). The random effect of tile ID explained 46.1% of the variation (random SD: 1.35; residual SD: 1.58).

	Estimate	SE	DF	*t*-value	*p*-value
intercept	−3.01	0.85	155	−3.55	**0.0004**
wave motion	1.34	0.93	154	1.44	0.1496
temperature	0.85	0.93	154	0.91	0.3611
days culture	2.32	0.31	155	7.53	**<<0.0001**
cover day 3	0.99	0.04	154	24.41	**<<0.0001**
wave motion:days culture	−1.33	0.35	155	−3.76	**0.0002**
temp:days culture	−0.94	0.35	155	−2.67	**0.0075**
				marginal R^2	**0.795**
				conditional R^2	**0.881**

Model formula: cover ~ wave motion + temperature + wave motion * days culture + temperature * days culture + cover at day 3, random = tile ID.

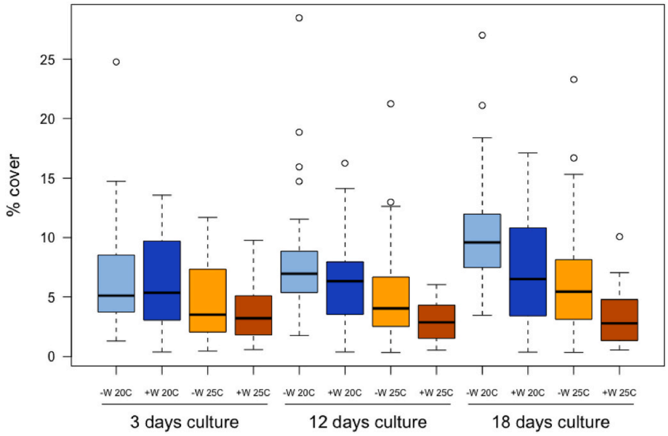

Fig. 1. Effects of temperature (20 °C vs. 25 °C) and wave motion treatment (without: W; with: +W) on percent cover of *E. amentacea* on the substrate medium (~20 cm^2 clay tiles) over time in culture.

4.00, $p = 0.0001$) and 18 d ($t_{309,} = 8.03$, $p = <0.0001$), respectively (Fig. 1). Cover increased significantly over time in culture, although low averages (2.5% increase from 3 to 18 d) can likely be accounted for by initial mortality of settled embryos. Finally, cover at the initiation of treatments (3 days after seeding) was the most significant predictor of growth in culture, comprising 69% of the variability accounted for by fixed factors (Table 2: marginal $R^2 = 0.795$ and 0.246 with *vs* without the inclusion of cover at 3 days as a covariate). Epiphyte cover was minimal and mostly consisted of small green algae, primarily found on the 20 °C treatment without water motion (Fig. 2A inset).

Field: Despite differences in initial cover at outplant (twice as high in 20 °C *vs* 25 °C; ANOVA $F(\text{temperature})_{1,155} = 35.1$, $p \ll 0.001$), growth in the field post-outplant was similar between tiles from each temperature treatment (Table 3; Fig. 2A). Seedlings cultured in 25 °C without waves grew rapidly, with no initial loss, attaining equivalent cover to the 20 °C treatments by 2 weeks, with final increases of 28.6% in 25 °C and 33.5% in 20 °C over four months (Fig. 2B). Cover on tiles which had been exposed to wave simulation showed initial drops with zero net growth over the first 4 weeks (Fig. 2A) but grew rapidly from 4 to 17 weeks. Apart from the driving role of cover at time of outplant and increases in cover over time ($p \ll 0.001$), factors representing clumping were the only significant predictors of field growth (maximum clump size, $p < 0.001$ and skew, $p = 0.028$; Table 3).

Table 3
Results of a generalized mixed model (beta model, logistic link) on the cover of *E. amentacea* over 17 weeks after implantation in the field (as proportion of tile substrate covered). Fixed factors include *temperature* (20 °C vs 25 °C) and *wave motion* (+/−) (all at 10% VS). Total cover of seedlings at the time of outplant (*initial cover*) and their distribution on the tile substrate (as represented by *max clump* size and *skew*) are modelled as covariates, while subsequent monitoring events (2, 4 and 17 weeks) form a fixed time factor *days*.

	Estimate	SE	*z*-value	*p*-value
intercept	−2.968	1.050	−2.83	**0.0047**
temperature	−0.025	0.042	−0.61	0.5441
wave motion	−0.308	1.403	−0.22	0.8262
days	0.018	0.001	14.44	**<<0.0001**
max clump	0.007	0.002	4.27	**<0.0001**
skew	0.085	0.039	2.20	**0.0280**
initial cover	0.121	0.028	4.40	**<0.0001**
temp:wave motion	−0.017	0.063	−0.27	0.7846

Model: cover ~ wave motion * temperature + days in field + initial cover + max clump + skew, random = tile ID.

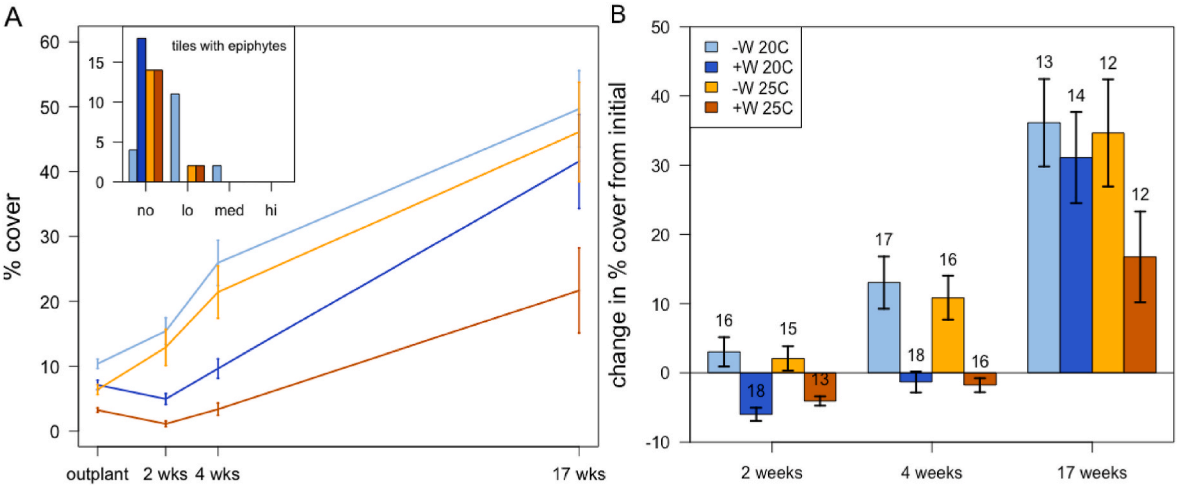

Fig. 2. Effects of temperature (20 °C vs. 25 °C) and wave motion (without: W; with: +W) on change in percent cover (means ± SE) over 17 weeks after implantation in the habitat to be restored, where **A)** shows absolute cover among treatments at each sampling event (2 weeks, 4 weeks and 17 weeks), and **B)** shows the growth (i.e. increase in cover from initial). The inset in **A)** depicts the number of tiles with relative levels of epiphyte growth at outplant: **no** = negligible growth; **lo** = little growth; **med** = medium epiphyte cover relative to **hi**, the maximum levels found). Numbers above bars in **B)** represent the number of tiles successfully located and surveyed.

3.3. Reduced nutrients and wave simulation

Culture: In the second experiment, wave motion increasingly reduced seedling growth in culture over time, independently of the culture medium nutrient content (*t*-ratios of 4.65 and 8.01 at 12 and 18 d and both $p < 0.0001$ in post-hoc comparisons following a significant wave-days interaction; Table 4). Consistent with the first experiment, cover of *E. amentacea* seedlings doubled from 3 to 18 days in the absence of waves (4.9–9.0%) but only increased by 50% with water motion (4.7–6.6%), although overall values remained low as before, with marginal increases over time and maximum cover reaching 28% (days in culture $p < 0.001$; Table 4; Fig. 3); post-settlement cover (at day 3) was again the most significant predictor of cover at the end of the culture period ($p \ll 0.001$; Table 4: marginal $R^2 = 0.765$ and 0.13 with *vs* without the inclusion of cover at day 3 as a covariate). In contrast, cover did not differ perceptibly with normal (VS) or reduced nutrient culture media (~10% VS; i.e. 500 + 30 *vs* 30 + 3 μM NO_3 + μM PO_4; Table 4). Higher nutrients did, however, promote the proliferation of epiphytes, but only in the absence of water motion, which strongly reduced epiphyte growth (Fig. 4A inset). Epiphytes were thus most abundant in the VS treatment without wave pumps (Fig. S1; Table S2), characterized by cyanobacterial growth in addition to the small green algae observed in other treatments.

Patterns of growth and epiphytes were consistent across a replicated culture event testing only the effects of culture medium nutrient levels (Von Stosch vs 10% VS, no wave pumps) in 2021 (Table S3; Fig. S2).

Field: At outplant, cover was 45% higher, on average, on tiles without wave motion in culture (ANOVA $F(\text{wave motion})_{1,155} = 19.6$, $p < 0.001$), with no difference contributed by culture nutrients ($p = 0.28$; Fig. 4A). In contrast, nutrients altered the early post-outplant negative impact of water motion on seedlings (GLMM nutrients: wave motion interaction, $p = 0.002$; Table 5; Fig. 4), such that higher nutrient levels in culture (VS) prevented initial mortality in the first two weeks in the native habitat (negative effect of waves at lower nutrients: $t = -4.70$, $p < 0.0001$ *vs* no effect with high nutrient levels). Wave-exposed tiles with reduced culture nutrients required four weeks to recover from initial losses. By 4 months, however, wave exposure had negligible remaining effects on growth ($p = 0.002$ *vs* $p = 0.92$ effects of waves at 2 *vs* 17 weeks; post-hoc comparison following significant wave-time interaction; Table 5; Fig. 4B). Cover at the time of outplant was also a significant predictor of field growth over time, as was maximum clump size (both $p < 0.001$). Overall, culture nutrients had no detectable independent effect on field growth, neither in 2020 (Table 5, Fig. 4) nor in a repeated experiment in 2021 (Fig. S3, Table S4).

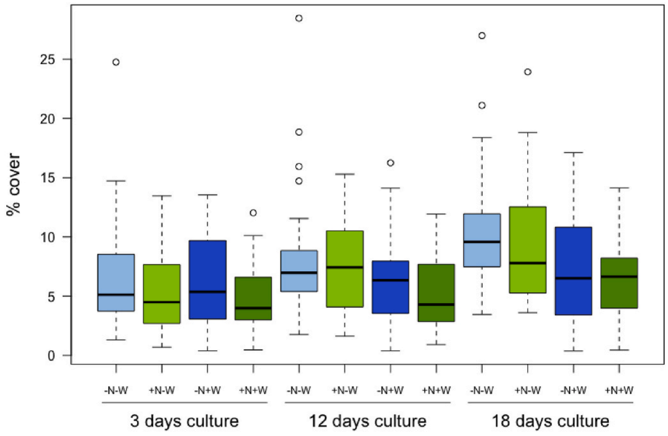

Fig. 3. Effects of nutrient and wave motion treatments on percent cover of *E. amentacea* on the substrate medium (~20 cm^2 clay tiles) over time in culture. Treatments were standard Von Stosch culture medium (+N) or reduced nutrients (10% VS; –N) with (+W) or without (–W) wave motion.

3.4. Oxidative stress in culture

Measures of oxidative stress and damage among culture media (Von Stosch *vs* reduced nutrient) were examined at the end of the culture phase in 2021. The content of ROS and the antioxidant enzymes SOD and CAT were elevated in seedlings cultured in VS relative to those with lower nutrient medium (*t*-test, all $p < 0.05$; Table 6). Conversely, there were no signs of lipid peroxidation (LPO), the indicator of oxidative damage (Table 6).

Table 4

Development of *E. amentacea* recruits as percent cover in culture (cover assessed at 3, 12 and 18 days) in treatments of standard Von Stosch or reduced nutrient (10% VS) culture medium with or without wave motion. *Cover at day 3* of culture was modelled as a covariate, while the other two dates form the fixed time factor *days culture*. (GLMM, gaussian family of residual distributions; assumptions checked and met with packages DHARMa and performance). The random effect of tile ID explained 37.0% of the variation (SD: 1.08; SD residual 1.84).

	Estimate	SE	DF	*t*-value	*p*-value
intercept	−2.88	0.96	155	−3.00	**0.0027**
nutrients	0.46	1.06	154	0.43	0.6649
wave motion	0.70	1.06	154	0.66	0.5096
days culture	0.37	0.06	155	6.19	**<<0.0001**
cover at day 3	1.02	0.04	154	26.14	**<<0.0001**
nutrients:days	−0.01	0.07	155	−0.07	0.9419
wave motion:days	−0.19	0.07	155	−2.76	**0.0058**
				marginal R^2	**0.765**
				conditional R^2	**0.825**

Model: cover ~ nutrients + wave motion + nutrients * days culture + wave motion * days culture + cover d3, random = tile ID.

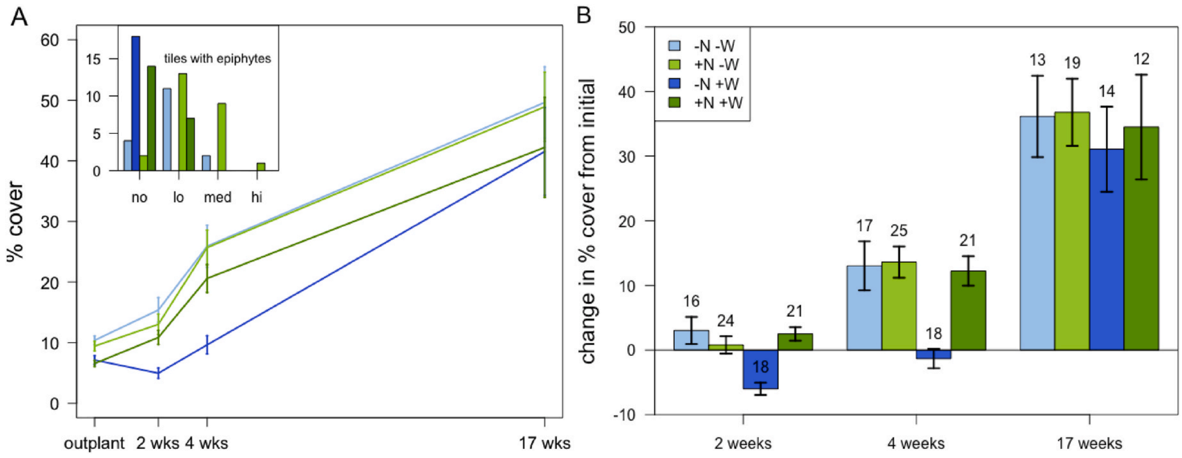

Fig. 4. Effects of nutrient reduction of culture medium (**+N:** 500 + 30 μM NO_3 + PO_4 *vs* **–N:** 30 + 3 μM NO_3 + PO_4) and waves (±) on change in percent cover over 17 weeks after outplant in the midlittoral habitat, where **A)** shows absolute cover among treatments at each sampling event (2 weeks, 4 weeks and 17 weeks) and **B)** depicts growth (i.e. increase in cover from initial). The inset in **A)** depicts the number of tiles with relative levels of epiphyte growth at outplant (as described in Fig. 2.). Numbers above bars in **B)** represent the number of tiles successfully located and surveyed.

Table 5

Generalized mixed model results on the cover of *E. amentacea* (i.e. proportion of tile substrate covered) over 17 weeks after outplant into the rocky midlittoral zone. Fixed factors include culture treatments of *nutrients* (standard *vs* reduced to 10% NO_3 + PO_4 Von Stosch culture medium) and *wave motion* (+/−) (all at 20 °C) as well as time (*days*: monitoring events at 2, 4 and 17 weeks). Covariates included cover at outplant (*initial cover*) and measures of seedling distribution on the tile substrate (*max clump* size and *skew*). The model was fit with a beta distribution and logistic link.

(A)	Estimate	SE	z-value	p-value
intercept	−2.78	0.27	−10.49	**<<0.0001**
nutrients	−0.30	0.20	−1.50	0.1338
wave motion	−1.17	0.23	−5.06	**<<0.0001**
days	0.02	0.00	14.09	**<<0.0001**
max clump	−0.01	0.00	−3.72	**0.0002**
initial cover	0.12	0.02	5.15	**<<0.0001**
nutrients:wave motion	1.25	0.29	4.32	**<0.0001**
waves:days	0.004	0.002	2.51	**0.0123**

Model formula: cover ~ nutrients * waves + waves * days + initial cover + max clump, random = tile ID.

Table 6

Physiological responses to different nutrient levels in the culture medium, where + N is Von Stosch medium with 500 μM NO_3 + 30 μM PO_4 and –N has 30 + 3 μM NO_3 + PO_4. ROS are reactive oxygen species and SOD and CAT are the enzymes superoxide dismutase and catalase involved in neutralizing ROS. LPO is lipid peroxidation, an indicator of the state of the antioxidant system.

Response	mean –N	mean +N	sd –N	sd +N	df	*t*-value	lower CI	upper CI	*p*-value
ROS AFU mg prot^{-1}	1.6×10^6	3.5×10^6	4.9×10^5	4.7×10^5	6.4	−5.93	-2.6×10^6	-1.1×10^6	**<0.001**
SOD U min^{-1} mg prot^{-1}	5.61	21.25	2.61	5.13	4.0	−5.32	−23.76	−7.52	**0.006**
CAT mmol min^{-1} mg prot^{-1}	0.020	0.035	0.006	0.010	6.7	−2.79	−0.027	−0.002	**0.028**
LPO nmol g^{-1} ww	131.5	132.8	11.3	19.9	7.9	−0.13	−22.81	20.39	0.90

3.5. Treatment effects on clumping and its role in initial field success

The effects of culture conditions on the development of clumped distributions of seedlings and their contribution to overall cover varied both among treatments and between experiments. In general, total numbers of polygons and clumping (as represented by maximum polygon size and the skew of the size distribution of polygons) were highest in treatments with highest cover (exp1: -W 20 °C; exp2: -N–W; Fig. S4) but differences among treatments were more pronounced in the first experiment (Table S5). Here, the contribution of clumping to total cover was strongly influenced by water temperature, with only marginal effects of wave motion (PERMANOVA *pseudo-*$F_{1,154} = 18.9$, $p = 0.001$ and *pseudo*$F_{1,154} = 2.2$, $p = 0.081$, respectively; Fig. 5A). Further examination of each size class revealed that differences lie in the smallest [0–2) and medium-large clumps [10–50), where the [0–2) size class, representing individuals rather than clumps, made up a higher portion of total cover in 25 °C treatments ($p << 0.001$) and marginally more in those with wave motion ($p = 0.044$). Medium-large clumps (both [10–30) and [30–50) polygons) contributed more to cover in 20 °C treatments ($p = 0.002$ and $\ll 0.001$, respectively; full results in Table S6).

A higher degree of clumping at the end of the culture period, in turn, played a strong positive role in *E. amentacea* survival in the first weeks as revealed by grouping ordinated clump sizes by the last confirmed date in which surviving individuals were found (tiles from all treatments in both experiments combined; Fig. 6). Survival groups (outplant, 28 d and 118 d) were separated along the NMDS1 axis, which was largely defined along a spectrum of smaller clump sizes (negative values) to larger ones (positive values).

In contrast, neither culture nutrients nor wave motion played a role in the development of clumps in culture in the second experiment (all *p*

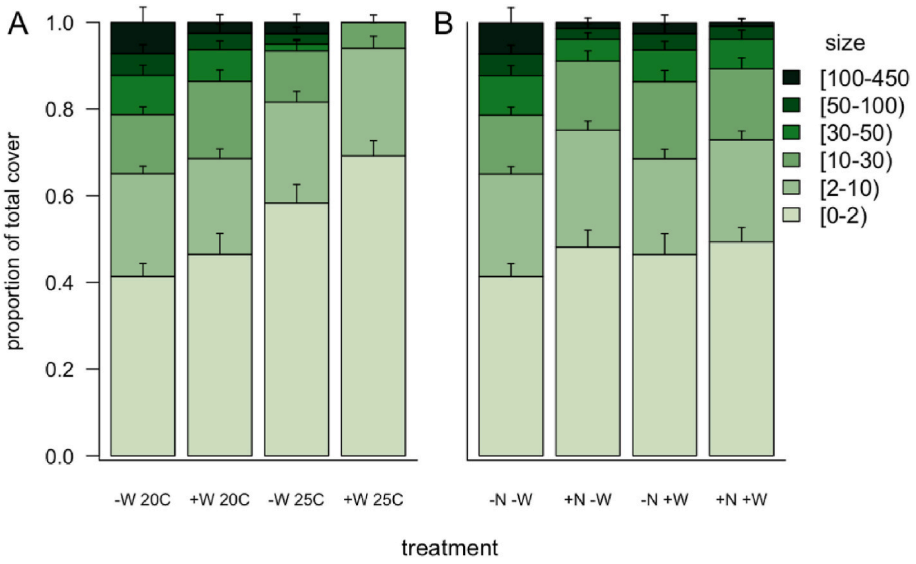

Fig. 5. Differences among treatments in the spatial distribution of *E. amentacea* seedlings across tiles at the end of the culture period (18 days), as represented by the proportion of total cover comprised by each of 6 size classes of polygons (mean ± SD in mm^2) obtained from image thresholding. **A)** Experiment 1: temperature (20 °C vs. 25 °C) and wave motion (without: -W; with: +W). **B)** Experiment 2: culture nutrient (+N: 500 + 30 μM N + P *vs* –N: 30 + 3 μM N + P) with (+W) or without (–W) wave motion. Larger polygon sizes indicate larger clumps.

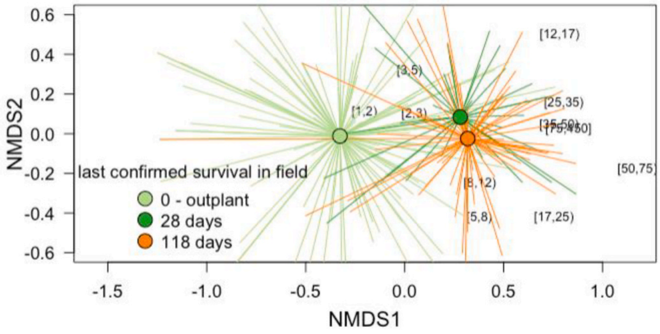

Fig. 6. NMDS of clump sizes of *E. amentacea* at the end of the culture period, grouped by minimum time of survival. Light green "0 – outplant" indicates tiles that were found without surviving algae or simply not found during any subsequent monitoring. Similarly, those in the 28-day group were not found with surviving algae after the 28-day monitoring event. Group 0 is separated by Groups 28 and 118 days along the NMDS1 axis, which is largely defined with the smallest clumps at the negative end and the largest clumps defining positive values.

> 0.1; Fig. 5B); however, the maximum clump size at outplant was a significant predictor of field growth (Table 5).

3.6. Extended monitoring (1–4 years)

While long-term monitoring was not the primary goal of this manuscript, it is essential for the evaluation of repopulation and restoration success and thus we report mid-term data here. Thallus lengths during the springtime period of maximal growth were 7.6 cm (3.1 SD, $n = 51$) in 2022 and 12.5 cm (2.2 SD, $n = 15$ tiles measured, randomly selected from 34 observed) in 2024 (Fig. S5). These data combine the outplants from 2020 to 2021, representing individuals one/three (2022) and two/four years' old (2024), which is reflected in the high variability. Summertime sampling of apices in 2021, 2022 and 2024 confirmed that both years' outplant populations reached reproductive maturity within one year of culture and remained fertile, with induced egg release observed in the laboratory (Fig. S6).

4. Discussion

4.1. Negative effects of increased temperatures and wave motion in culture do not translate into impacts on field outcomes

Consistently warm temperature in culture (25 °C) and exposure to wave motion independently impeded seedling growth, beginning from early days in culture and increasing over time (Table 3; Fig. 2). In contrast, no effects were visible on field growth after the transition period; even those tiles with initial post-outplant losses in cover attained similarly high cover after four months.

Thermal tolerance in seedlings of fucoid species is highly correlated to the reproductive period (Steen and Rueness, 2004; Steen and Scrosati, 2004), where adults are more physiologically plastic and show a higher thermal tolerance (Graiff et al., 2015; Schmid et al., 2021) than their environmentally sensitive early life stages (Brawley and Johnson, 1991; Johnson and Brawley, 1998). Accordingly, work on the intertidal species *Ericaria giacconei* showed that temperatures above the mean during their wintertime reproductive period (~15 °C) retarded development or caused recruit mortality, while adults showed no physiological stress to temperatures up to 28 °C (Falace et al., 2021). Yet, gamete release and zygote settlement in *E. amentacea* generally occur in late June or July when water temperatures average 23–25 °C with greater extremes in the intertidal resulting in mean daily highs of nearly 30 °C (R. Clausing, unpubl. data). This suggests that 25 °C may be physiologically appropriate for cultivation of the species. Indeed, previous work with *E. amentacea* showed that germling survival was equivalent at both 20 °C and 24 °C until epiphyte proliferation at the warmer temperature inhibited normal development (Falace et al., 2018). Reconciling temperature and contamination is a common problem in macroalgal aquaculture (Visch et al., 2023). Yet, temporary exposure to increased temperatures (~12 h at 25–26 °C every 3 days) was found to improve culture growth of *E. amentacea* seedlings (Clausing et al., 2022). This apparent discord with the present results may potentially relate to temperature-induced upregulation of phenol production, secondary metabolites known to protect again epiphytes as well as grazing and UV radiation, where total phenol content in *E. amentacea* has been found to increase with short-term heat exposure (8–24 h at 25 °C, as in Clausing et al., 2022) but is dramatically reduced with chronic exposure (>48 h) (Mannino et al., 2016).

The absence of temperature treatment effects in the field likely relate to a limited duration or lack of physiological effects from culture priming at 25 °C, combined with the rapid adaptation of algae to field conditions. Further, epiphyte cover was uniformly low, despite expectation of enhanced overgrowth at higher temperatures (Falace et al., 2018), likely resulting from the universal use of reduced nutrient culture media (~10% VS); this absence of differential negative effects associated to epiphyte overgrowth also likely contributed to similar patterns of increase in cover in the field.

The highly negative effects of wave motion on seedling development over time in culture (Table 2; Fig. 1) were unexpected, given the hydrodynamic nature of the native habitat and positive effects previously observed on other *Cystoseira s.l.* species (*G. barbata* in field mesocosms: Lokovšek et al., 2023). One cause of the negative impacts may be the timing of implementation. While *E. amentacea* recruits are exposed to extreme water motion in the field (Thibaut et al., 2014), it is likely that zygotes settling within adult populations are protected from maximal flow rates or that only those in microrefugia survive, as wave stress is a main driver of early post-settlement mortality in intertidal brown algae (Vadas et al., 1990, 1992). In a study showing benefits of water motion on cultured *Cystoseira s.l.*, wave pumps were initiated on older algae (3 months: Lokovšek et al., 2023), suggesting that effects might be different if the wave motion treatment was initiated on later life stages maintained longer in culture. Alternatively, algal cover as the response variable may not best represent the benefits provided by exposure to hydrodynamics, as brown algae in exposed environments have been shown to increase in toughness while attaining smaller size then their counterparts in more sheltered waters (Gaylord et al., 1994). Higher allocation to structural components with water motion (Kraemer and Chapman, 1991; Hurd, 2000) may reduce overall rates of growth, but reinforce rhizoidal attachment to the substrate, which is highly adaptive in an exposed environment.

The absence of positive or negative impacts of culture wave exposure on field growth is likely explained by the fact that the water motion produced in the aquaria is not sufficiently intense to serve as a proxy of the shear stress and wave shock they experience in the intertidal habitat. Organisms inhabiting the surf zone of exposed shores may experience exceptionally high water velocities and accelerations (Gaylord et al., 1994) associated with turbulence and resulting drag and impingement forces (Denny and Gaylord, 2002). Thus, the waves produced by the pumps may insufficiently replicate the forces exerted on algae in the sea to induce the same physiological responses that could alter patterns of growth in the initial days post-outplant.

4.2. Nutrient levels do not alter growth in culture but mediate effects of wave motion in initial post-outplant resilience

The reduction of nutrients by 90% from the standard culture media had no apparent independent effects on the development of *E. amentacea* cover in culture, nor in the field. With concentrations (μM) of 500 + 30 and 30 + 3 $NO_3 + PO_4$, both culture media are high-nutrient relative to the oligotrophic native environment on the Ligurian coast and provide similar relative N:P availability (~15:1), making nutrient limitation in culture unlikely. Although data indicates that production in intertidal *Cystoseira s.l.* species becomes N-limited in summer due to thermal stratification (Celis-Plá et al., 2016, 2017), *Cystoseira s.l.* species have the capacity to store N and P in their tissues in response to availability (Delgado et al., 1994), with increases up to 50% in tissue N content with N-enrichment (Celis-Plá et al., 2014). Taken together, this suggests that algal tissue nutrient stores at the end of the culture period were replete in both treatments, and thus nutrient reduction conferred no direct disadvantage with transfer to the low-nutrient habitat.

In contrast, nutrient reduction did interact with water motion to entirely inhibit epiphyte growth in culture (–N + W treatments), where epiphyte proliferation (medium or high cover) occurred almost exclusively in VS medium without wave simulation. With capacity for rapid, often unsaturated nutrient uptake, high nutrient levels may confer a competitive advantage to epiphytic algae over perennial species with lower uptake rates, such as *E. amentacea* (Fujita, 1985; Lobban and Harrison, 1997; Pedersen and Borum, 1997; Lotze and Schramm, 2000). Epiphyte overgrowth in turn may severely compromise seedling fitness, reducing photosynthetic efficiency by physically blocking light and carbon uptake on the thallus surface (Sand-Jensen, 1977; Sand-Jensen and Borum, 1991). Further, competition for light may prioritize upward growth of seedlings, reducing resource allocation to rhizoidal reinforcement (Malfatti et al., 2023).

Even in high-nutrient culture media, high flow rates with wave pumps strongly reduced epiphyte colonization (+N + W); moreover, the same mechanical forces may further benefit the algae by inducing augmented structural reinforcement (Kraemer and Chapman, 1991) and rhizoidal growth (Fletcher and Callow, 1992) as well as increasing water oxygenation and reducing the diffusion boundary layer, improving gas and nutrient uptake on the thallus surface (Hurd et al., 1996; Harrison and Hurd, 2001). Thus, the initial post-outplant losses observed in the –N + W treatment were unexpected. They suggest the presence of tertiary factors that mediate the interplay of nutrients and water motion with algal physiology and susceptibility to the environmental stressors of the habitat (Celis-Plá et al., 2014, 2016), such as seedling density (fucoid seedlings: Creed et al., 1997) or treatment-associated differences in the algal microbial community (Wahl et al., 2012; Mancuso et al., 2023).

Despite the initial losses in the -N + W treatment, it is important to note that, like in the first experiment, tiles with surviving seedlings after the first weeks post-outplant were able to attain similarly high cover after four months in the intertidal habitat. Thus, given the strong negative impacts of epiphytes on seedling fitness, these results suggest that nutrient reduction may be an important strategy to improve seedling survival at outplant. Moreover, evidence that nutrient enrichment can disrupt the microbiome of *Cystoseira s.l.* and augment disease (Mancuso et al., 2023) provides additional incentive to reduce nutrients to more realistic concentrations. Finally, we suggest that more research on the use of waves is necessary, testing alternate measures of growth and toughness (e.g. rhizoidal growth, thallus toughness and alginic acid content) as well as later onset of waves after embryo settlement.

4.3. Lack of oxidative damage in response to high nutrients indicate an antioxidant system capable of managing stress

Parameters of oxidative stress and damage were used to further investigate any biochemical consequences resulting from different nutrient content in the culture media, as effects were not observable by measures of algal cover alone. While essential biological functions in macroalgae may produce low levels of ROS (e.g. photosynthesis: Dummermuth et al., 2003), the higher ROS content in seedlings cultured in high-nutrient VS reveal the onset of oxidative stress. The parallel increases in the enzymatic antioxidants SOD and CAT indicate the activation of an antioxidant machinery evolved to protect cells against potential ROS damage, including disruption of cellular structures such as DNA, proteins and lipids (Rezayian et al., 2019).

One possible cause of the nutrient-induced oxidative stress may be the increased occurrence of epiphytes, as has been observed in field-collected *Dictyota* with or without epiphytes (Tejada and Sureda, 2014). Alternatively, the addition of nutrients may have increased photosynthetic efficiency and resulting antioxidant activity, as has been hypothesized for *E. selaginoides* and red macroalgae subjected to nutrient enrichment (Celis-Plá et al., 2014; Long et al., 2024). Regardless the mechanism, the lack of lipid peroxidation associated to increases in ROS and SOD and CAT activities show that the antioxidant system was effectively induced and able to prevent the onset of cellular damage in this early life stage. Additionally, given that oxidative stress in macroalgae is predominantly investigated in the context of environmental (thermal, UV, salinity, etc) stress or pollutants (Santos et al., 2012;

Figueroa et al., 2014; Maharana et al., 2015; Zhang et al., 2020) and that data for early life stages is generally lacking (Contreras et al., 2007; Gong et al., 2018), these results may have additional significance for algal cultivation for commercial purposes.

4.4. Seedling clumping, driven by zygote settlement, improves field survival beyond initial weeks

Greater clumping of individuals was associated with greater long-term survival (beyond 4 weeks, Fig. 6) and growth in the native habitat, regardless of total cover at outplant. Measures of aggregation were stronger predictors than culture treatments in both experiments, demonstrating a driving role that is consistent with recent restoration-orientated work on this species (Clausing et al., 2022) and on other species inhabiting high-stress environments (Silliman et al., 2015; Duggan-Edwards et al., 2019; Renzi et al., 2019). Thus, the lack of large aggregations of individuals in the 25 °C wave-exposed treatment is a likely hypothesis for its reduced performance. Both overall cover and clumping in turn, are driven by initial zygote settlement, which is strongly linked to the quantity and quality of reproductive material available. This highlights the importance of phenological studies and monitoring to optimize the period of collection (Bevilacqua et al., 2019; Cimini et al., 2024).

4.5. General considerations to improve restoration potential

The goal of this study was to investigate the effect of priming during cultivation on seedling resilience, via coverage, to the environmental conditions of the habitat over the first few months in the sea. While successful embryo settlement and seedling survival and establishment during the transition to the field represent the first bottleneck to restoration success (Vadas et al., 1992), they do not guarantee that algae survive long-term and repopulate. Yet, continued monitoring (4 years) demonstrated that algae that survive the first 4 months grow to full tile coverage seemingly without exception (i.e. no empty tiles found on the rock), where tile detachment from the benthos is the only observed source of mortality. Moreover, the observation of egg release from both years' outplants in the summers of 2022 (1–2 years old) and 2024 (3–4 years old) indicate the potential for self-repopulation of the neighboring area, which may be evident on a longer term (over 10 years: Gran et al., 2022). With the aim of improving the *ex-situ* outplanting technique to scale up restoration actions, further research on substrate materials and methods of attachment are essential to reduce tile loss over time.

While increased temperature and wave motion negatively affected seedling growth in culture, and higher nutrients caused oxidative stress likely associated with enhanced epiphyte overgrowth, these effects were not clearly translated into field outcomes: survival and cover at 4 months were similar among all treatments. Given the complex array of environmental stressors and their stochasticity in the intertidal habitat, these results suggest that there may be a size threshold that confers resilience to environmental extremes. Moreover, they underscore the adaptability of these algae and the need for a strong base of knowledge on the physiology of early life stages to develop and implement a successful *ex-situ* restoration action.

We suggest that reducing NO_3 and PO_4 in the culture media may be an adaptive approach to reduce epiphytes, but investigation into higher N:P ratios or other N sources is still needed (Malfatti et al., 2023). Potential benefits of exposure to wave simulation and higher culture temperature remain to be clarified, and likely depend on the timing of delivery (e.g. waves initiated after a certain point of rhizoidal development and pulse vs. press heat exposure). Moreover, alternative measures to algal cover are likely needed to detect conferred benefits. Dedicated culture experiments that incorporate measures of thallus strength and physiological stress and performance together with other metrics of growth may provide insight into seedling development and growth strategy, allowing for more targeted selection of culture conditions that enhance seedling fitness in the native environment.

Overall, the present work stresses the importance of 1) the zygote settlement phase to establish critical densities of seedlings that confer self-facilitative benefits in the post-outplant phase and 2) a more realistic, less-controlled culture approach that both reduces human resource costs in culture and introduces environmental variability and mild stress to improve survival during the transition to the sea. Given the bottleneck of initial survival and the evidence that, after 4 months, mortality is low and algae continue to grow and attain reproductive maturity, addressing these points and additionally improving means of substrate attachment to the rock may ensure restoration success and scalability of the approach.

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CRediT authorship contribution statement

Rachel J. Clausing: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Annalisa Falace:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Gina De La Fuente:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Camilla Della Torre:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Mariachiara Chiantore:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Valentina Asnaghi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106718.

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