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3 **Addressing reproductive stochasticity and grazing impacts in the restoration of**  
4 **a canopy-forming brown alga by implementing mitigation solutions**  
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For Peer Review

## 1 Abstract

- 1 2 1. The worldwide decline of marine forests, due to human impacts and climate change,  
3 4 emphasizes the need to develop and implement effective and sustainable solutions to restore  
5 6 these endangered habitats and to re-establish the services they provide.  
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- 9 10 2. In this study, the *ex situ* restoration of *Treptacantha barbata*, a Mediterranean subtidal habitat-  
11 12 forming species of brown seaweed, was for the first time implemented in a marine protected  
13 14 area in the Adriatic Sea. Two restoration efforts were performed in 2019. The first one was  
15 16 started in winter, after a marine heatwave that triggered early fertility, the second one in  
17 18 spring, when the species usually reproduces.
- 19 20 3. This study aimed to evaluate: 1) the disruptive effects of a thermal anomaly on the  
21 22 reproductive biology and performance in culture of *T. barbata*; and 2) the impact of the  
23 24 grazing pressure on juveniles after the outplanting.
- 25 26 4. The first cultivation was more productive than the second one, in terms of zygote release and  
27 28 germling growth. To mitigate the low efficiency of the second culture and to avoid prolonged  
29 30 highly-demanding maintenance in the mesocosms, the cultivation period was extended  
31 32 outdoors using a structure suspended in the water column.
- 33 34 5. The modular frames conceived for outplanting *T. barbata* proved to be effective because of  
35 36 their easy operability and low cost. Controlling for herbivorous fish had significant positive  
37 38 effects on both juvenile survival and growth.
- 39 40 6. The outcomes highlighted that an unpredictable climatic event and fish grazing were major  
41 42 threats that impaired the restoration process of *T. barbata*. These stressors should be  
43 44 considered when developing plans to implement effective large-scale restoration of canopy-  
45 46 forming macroalgae.  
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49 25 **KEYWORDS:** climate change, early life stages, *ex situ* restoration, fucoid, macroalgae, marine forests, heatwave,  
50 26 phenology, subtidal.

## 51 1. INTRODUCTION

52 28 In the last four decades, the decline of *Cystoseira sensu lato* (henceforth referred to as *Cystoseira*)  
53 29 (Fucales, Phaeophyceae) forests in the Mediterranean Sea has been widely documented (e.g. Munda,  
54 30 1980; Munda, 1982; Hoffman et al., 1988; Munda, 1993a; Cormaci & Furnari, 1999; Thibaut et al.,  
55 31 2005; Mangialajo, Chiantore & Cattaneo-Vietti, 2008; Thibaut et al., 2015a; Thibaut et al., 2015b;  
56 32 Blanfunè et al., 2016; Valdazo et al., 2017; Mancuso et al., 2018) and has been mainly attributed to  
57 33 the interplay of several human impacts on the coastal environment, such as habitat destruction, and  
58 34 pollution together with overgrazing by sea urchins and herbivorous fish (e.g. Bellan-Santini,  
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3 35 1966; Arnoux & Bellan-Santini, 1972; Munda, 1974; Munda, 1982; Verlaque, 1987; Hoffman et al.,  
4 36 1988; Munda, 1993b; Chryssovergis & Panayotidis, 1995; Cormaci & Furnari, 1999; Benedetti-  
5 37 Cecchi et al., 2001; Soltan et al., 2001; Hereu, 2004; Sales & Ballesteros, 2009; Sales et al., 2011;  
6 38 Gianni et al., 2013; Pinedo, Zabala & Ballesteros, 2013; Strain et al., 2014). Recent studies have also  
7 39 suggested or reported the disruptive effect of climate change on the distribution, abundance and  
8 40 biology of these species (e.g. Buonomo et al., 2018; Bevilacqua et al., 2019; Cáliz et al., 2019;  
9 41 Capdevila et al., 2019).

15 42 *Cystoseira* forests provide critical ecosystem services in littoral habitats by increasing the complexity  
16 43 of rocky bottoms and thus supporting rich biodiversity and complex food webs (e.g. Sauvageau,  
17 44 1912; Funk, 1927; Boudouresque & Lück, 1972; Giaccone, 1973; Ballesteros, 1988; Rull & Gómez  
18 45 Garreta, 1989; Otero-Schmitt & Pérez-Cirera, 1996; Ballesteros et al., 1998; Pizzuto, 1999;  
19 46 Ballesteros et al., 2009; Cheminée et al., 2013; Pitacco et al., 2014; Chiarore et al., 2019; Cuadros et  
20 47 al., 2019).

25 48 Due to their compromised status and key ecological role, all Mediterranean *Cystoseira* species  
26 49 (except *C. compressa*) are included on the “List of endangered or threatened species” of the Barcelona  
27 50 Convention (amended Annex II of the “Protocol concerning Specially Protected Areas and biological  
28 51 diversity”; UNEP, 2019), and some species are “strictly protected” by the Bern Convention  
29 52 (Appendix I; Council of Europe, 1979). In addition, *Cystoseira* assemblages are considered habitats  
30 53 of critical importance in the EU (Directive 92/43/EEC; Annex I, included in “Rocky reefs” – Council  
31 54 of Europe, 1992) and are indicators of the good ecological status of coastal waters in the context of  
32 55 the Water Framework Directive (2000/60/EC) (i.e. CARLIT – Ballesteros et al., 2007; Blanfunè et  
33 56 al., 2017; EEI – Orfanidis, Panayotidis & Stamatis, 2003).

41 57 Although establishing marine protected areas (MPAs) can reduce some of the human impacts on these  
42 58 forests, it is insufficient to ensure their resilience (Gianni et al., 2013; Medrano et al., 2020b). To  
43 59 date, there has been little evidence of the natural recovery of degraded *Cystoseira* stands (e.g. Munda,  
44 60 2000; Iveša, Djakovac & Devescovi, 2016; Orlando-Bonaca & Rotter, 2018; Medrano et al., 2020b)  
45 61 because, when large-scale losses have occurred, replenishment from nearby populations is rather  
46 62 difficult due to the short-distance dissemination of the eggs/zygotes and the low population  
47 63 connectivity (e.g. Soltan et al., 2001; Buonomo et al., 2017; Capdevila et al., 2018).

53 64 For these reasons, active restoration of *Cystoseira* forests has been recommended, and different  
54 65 methods have been tested, such as the transplantation of adult thalli (e.g. Falace, Zanelli & Bressan,  
55 66 2006; Susini et al., 2007; Perkol-Finkel et al., 2012) and recruitment enhancement, both *ex situ*  
56 67 (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019; Medrano et al., 2020a) and *in*  
57 68 *situ* (Verdura et al., 2018; Medrano et al., 2020a). The implementation of the recruitment

69 enhancement showed positive outcomes and is preferable because it is more sustainable and has a  
70 lower impact on donor populations than adult transplantation.

71 Nevertheless, some knowledge gaps and bottlenecks hinder large-scale *Cystoseira* restoration, and  
72 more research is needed to develop effective, sustainable and cost-effective solutions. Improving  
73 species-specific cultivation protocols could maximize germling survival, given the multiple stressors  
74 that can cause mortality in the early developmental stages (e.g. Dudgeon & Petraitis, 2005; Schiel &  
75 Foster, 2006; Irving et al., 2009; Araujo et al., 2012). Optimal culture conditions can also maximize  
76 germling growth (De La Fuente et al., 2019) and support the attainment of a “refuge” size (Vadas,  
77 Johnson & Norton, 1992), which reduces the consumption and bull-dozing impact of macro- and  
78 mesograzers (e.g. crustaceans, molluscs) in the outplants. However, grazing by fish and sea urchins  
79 frequently represents a major threat to the survival and growth of outplanted organisms (e.g.  
80 Mangialajo et al., 2012; Perkol-Finker et al., 2012; Gianni et al., 2013; Ferrario et al., 2016; Gianni  
81 et al., 2018; Gianni et al., 2020; Medrano et al., 2020a). Verlaque (1990) reported that up to 60% of  
82 the gut contents of *Sarpa salpa* can be made up of *Cystoseira*. This herbivore can remove up to 90%  
83 of the surface area of transplanted adults in a few days (Gianni et al., 2018) and sometimes even in a  
84 few hours (Gianni et al., 2017), completely nullifying reforestation efforts. Finally, extreme climatic  
85 events (i.e. thermal anomalies, storm surges) can alter the reproductive timing of species (Bevilacqua  
86 et al., 2019) or hamper the survival/growth of outplanted juveniles (De La Fuente et al., 2019).

87 The present study, developed within the framework of the European project ROCPOPLife (LIFE16  
88 NAT/IT/000816), focuses on the restoration of *Treptacantha barbata* (= *Cystoseira barbata*) by  
89 recruitment enhancement in the Miramare MPA (Gulf of Trieste, Northern Adriatic Sea). This species  
90 thrives in shallow coastal waters with low hydrodynamism and is fairly tolerant of eutrophic  
91 environments (e.g. Feldmann, 1937; Montesanto & Panayotidis, 2001; Sfriso, Curiel & Rismondo,  
92 2009; Falace et al., 2010).

93 In large areas of the Gulf of Trieste, the distribution of *T. barbata* has declined in the last 30 years  
94 (Falace & Bressan, 2003; Falace, Di Pascoli & Bressan, 2005; Falace et al., 2010) to the point that *T.*  
95 *barbata* is now only present along the Slovenian coast (Orlando-Bonaca & Rotter, 2018). It is worth  
96 noting that, despite extensive monitoring, the species was not found to be fertile in 2017 and 2018,  
97 while it was found to be fertile twice in 2019: first in winter, prematurely, after a marine heatwave  
98 (MHW) (Bevilacqua et al., 2019), and then in spring, when it usually reproduces (Falace & Bressan,  
99 2006). Both times, *ex situ* restoration techniques were used, which represents the first implementation  
100 of *T. barbata* restoration in this geographical area.

101 Overall, this study aimed to evaluate the following:

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1) the effects of a thermal anomaly on the reproductive biology and performance in culture of *T. barbata* (the first phase of *ex situ* restoration), and

2) the impact of fish grazing pressure on juveniles in the first, most critical, months following outplanting (the second phase of the *ex situ* restoration).

The outcomes of these restoration efforts led to the implementation of mitigation solutions to overcome some of the bottlenecks in subtidal canopy-forming macroalgae restoration (i.e. reproductive stochasticity, fish grazing) from the perspective of upscaling these restoration techniques.

## 2. METHODS

### 2.1 Study sites

The donor and the receiving sites are both located in the Gulf of Trieste, the northernmost part of the Adriatic Sea (Eastern Mediterranean), approximately 30 km apart (Figure S1). The source of fertile material was Strunjan Natural Park in Slovenia (45.535105, 13.628437), which is characterized by healthy *Cystoseira* forests in which *T. barbata* occurs in association with *Cystoseira compressa* and *Carpodesmia crinita*. The restoration site was the Miramare MPA in Italy (45.701835, 13.714113), where *T. barbata* was once present (Bussani & Vukovic, 1992) but has not been observed in the last 20 years and only isolated thalli of *C. compressa* can be found (Falace, Di Pascoli & Bressan, 2005). The cultured germlings were outplanted in the “no-take” area of the Miramare MPA (30 ha out of the total 120 ha), where human activities are prohibited.

### 2.2 Laboratory *ex situ* cultivation

The *ex situ* cultivation protocol of Falace et al. (2018), as improved by De La Fuente et al. (2019), was applied for the first time to *T. barbata*. Fertile apices were harvested in Strunjan Natural Park and transported under dark and cold (4 °C) conditions to the University of Trieste facilities within 1 hour (Figure S2). Then, the apices were rinsed with filtered sea water and stored in dark conditions at 4 °C for 36 hours. Under controlled culture conditions, clay tiles (4.5 cm in diameter, with a 0.6 cm hole at the centre to screw the tile onto the outplanting modular supports, total area of 12.65 cm<sup>2</sup>) were seeded with five apices each in 8-L aquaria. The light intensity was set at 125 μmol photons m<sup>-2</sup> s<sup>-1</sup> with a photoperiod of 15:9 hours light:dark, and the temperature was set at 18 °C. These conditions were set to resemble those in which the species typically reproduces in the Gulf of Trieste (considering the reference period April-May, as reported in Falace & Bressan, 2006). The medium (Von Stosch’s enriched filtered sea water) was renewed every three days to minimize any possible effect of nutrient limitation and was continuously aerated through bubbling and pumping

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3 135 (approximately 300 L h<sup>-1</sup> flow) to ensure that the medium was oxygenated. The culture period lasted  
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5 136 four weeks.

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7 137 This protocol was applied in two separate cultures in 2019 as described below.

### 8 138 **2.3 First *ex situ* cultivation and outplanting**

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10 139 In February 2019, an MHW (+2.65 °C than the average monthly sea-surface temperature during the  
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12 140 last 40 years) occurred in the Northern Adriatic Sea, causing a phenological shift that abruptly  
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14 141 triggered fertility in *T. barbata* at the donor site (Bevilacqua et al., 2019). At the end of February, the  
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16 142 thalli still showed a winter *habitus* without well-developed primary branches (Figure 1a,b) such that  
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18 143 the receptacles grew on the adventitious branches (Figure 1c). The receptacles (Figure 1c,d) were  
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20 144 smaller than those that typically develop in spring on the primary branches (Figure 1e,f,g,h); thus,  
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22 145 some of the receptacles were sampled to test whether they were able to release viable zygotes.

23 146 Since the sampled receptacles released zygotes that developed into viable embryos, in March,  
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25 147 approximately 2500 fertile apices of *T. barbata* were harvested, and 420 clay tiles were seeded.  
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27 148 Twenty-four hours after seeding, the apices were removed, and this time was considered the time of  
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29 149 fertilization (T<sub>0</sub>).

30 150 During the laboratory culture, data were collected and processed as follows (Table S1):

- 31 151 – Thirty tiles were randomly selected and tagged at T<sub>0</sub>. These tiles were used for: (1) calculating  
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33 152 the *release efficiency* at T<sub>0</sub> as the *no. zygotes released per tile/no. receptacles per tile*, and (2)  
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35 153 calculating the germling survival rate between two (T<sub>1</sub>) and four (T<sub>2</sub>) weeks after fertilization  
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37 154 as the *% survival = no. germlings per tile at T<sub>2</sub> x100/no. germlings per tile at T<sub>1</sub>* through  
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39 155 photographic sampling at T<sub>1</sub> and T<sub>2</sub> with a Nikon D300 camera (Nikon Corporation, Tokyo,  
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41 156 Japan).
- 42 157 – One hundred and twenty tiles were randomly selected at both T<sub>1</sub> (Figure S3a) and T<sub>2</sub> (Figure  
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44 158 S3b) and photographed with a Nikon D300 camera (Nikon Corporation, Tokyo, Japan) to  
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46 159 assess germling density (i.e. the number of germlings per tile).
- 47 160 – Twenty tiles were randomly selected for morphometric measurements at T<sub>2</sub>. All the germlings  
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49 161 were carefully removed from the tiles, and 1,200 of them were selected at random and observed  
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51 162 under a stereomicroscope (Leica MZ 6, Leica Microsystems, Wetzlar, Germany), where they  
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53 163 were photographed with a Nikon Coolpix 4500 camera (Nikon Corporation, Tokyo, Japan)  
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55 164 (Figure S4).

56 165 The number of zygotes released per tile ( $n = 30$ ), the number of germlings per tile at T<sub>1</sub> and T<sub>2</sub> ( $n =$   
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58 166 120), and the germling length at T<sub>2</sub> ( $n = 1,200$ ) were obtained by processing photographs with ImageJ  
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60 167 software (Schneider, Rasband & Eliceiri, 2012). Specifically, counts were performed visually by  
60 168 superimposing a grid on each photograph and counting the number of items in each grid cell.



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3 169 Additionally, to describe the embryology of *T. barbata*, several glass slides were seeded. Zygote and  
4 embryo development were studied with an inverted microscope (Leica DM IL LED, Leica  
5 Microsystems, Wetzlar, Germany), and photographs were taken with a Canon Powershot G9 camera  
6 (Canon Inc., Tokyo, Japan) (Figure S5).  
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10 173 In the last four days of culture before outplanting (in April 2019), the temperature was gradually  
11 lowered to 13 °C to acclimate the germlings to the measured field temperature. Thereafter, 400 tiles  
12 were transported to the Miramare MPA (20 minutes by car) in refrigerated boxes filled with filtered  
13 sea water and attached with screws to 14 previously assembled metal modular frames in  
14 approximately four hours. Each modular frame consisted of one 100 x 2.5 cm flat perforated bar made  
15 of galvanized iron with four identical 50 x 2.5 cm bars attached perpendicular to the first bar to form  
16 a rake-like shape (Figure 2). Each tile was fastened to the bar with a bolt at a fixed distance of  
17 approximately 5 cm from the other tiles, for a total of approximately 28 tiles per modular structure.  
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20 181 The modular frames were specifically designed to mimic the spatial distribution pattern of *T. barbata*  
21 adult individuals at the donor site. Therefore, by using photoquadrats, we gathered information about  
22 the density (i.e. 43 thalli m<sup>-2</sup> ± 5, average ± SE) and mean adult thallus diameter (i.e. 15 cm ± 0.12)  
23 in the patches at the donor site. The final structure of the modular frames had a capacity of 30 tiles  
24 per frame, assuming the survival of one thallus per tile to the adult stage.  
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27 186 Two types of modular frame were conceived for testing and mitigating the effects of fish grazing  
28 (grazing trial): a basic frame and a frame with deterrents against herbivores (Figure 2a,b). Each  
29 deterrent was made of two flexible metallic mesh strips (3.5 x 20 cm, 0.5 cm mesh size) crossed at  
30 their midpoints to form an “X” shape (Figure 2c). The ends of the strips had a spike shape to deter  
31 fish. The overlapping strips were placed under the tiles before attaching them to the substrate with  
32 screws. The protective structure was formed by bringing the ends of the strips around the tile like the  
33 petals of a flower, creating a cage-like structure around the tile.  
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43 193 Of the 400 tiles, half were affixed to the metallic modular frames without protection (“Unprotected”,  
44 *UP*) (Figure 2a), and the remaining 200 tiles were protected with grazing deterrents (“Protected”, *P*)  
45 (Figure 2b). The 14 modular frames (7 *UP*, 7 *P*) were randomly affixed with screws to the rocky  
46 bottom at 3 m depth, the same depth at which *T. barbata* thrives in the donor population.  
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49 197 In each treatment (*UP*, *P*), thallus length was measured on 30 randomly selected tiles at 15 days (Time  
50 G1, where G refers to the grazing trial), 30 days (Time G2), 60 days (Time G3), 90 days (Time G4),  
51 120 days (Time G5) and 180 days (Time G6) after tile deployment. At Time G6, the number of tiles  
52 on which juveniles were present and the respective number of juveniles per tile were counted.  
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56 201 Bimonthly, the grazing deterrents were gently brushed with a wire brush to remove sediment and  
57 epiphytes that could decrease the light availability on the tiles and thus limit juvenile growth.  
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3 203 In June, July, and August 2019, the density of *S. salpa*, an herbivorous fish that typically grazes on  
4 204 *Cystoseira* (Verlaque, 1990), was assessed with a strip-transect visual census (Brock, 1954) inside  
5 205 the “no-take” zone of the MPA. Because of the limited dimensions of this zone, the method was  
6 206 modified, and the counts were performed simultaneously by two scuba divers along two parallel  
7 207 transects of approximately 1.2 km at different depths. In addition, to document the activity of grazers  
8 208 on the germlings, an underwater camera (GOPRO Hero8 Black Edition, GoPro, San Mateo,  
9 209 California) was affixed between some of the modular frames. Video sampling was carried out every  
10 210 3 weeks during the experiment.

#### 17 211 **2.4 Second *ex situ* cultivation and outplanting**

18 212 The second cultivation effort began in May 2019. During receptacle collection in Strunjan Natural  
19 213 Park, the donor population appeared to be depleted of its reproductive potential, with small  
20 214 receptacles (Figure 1d), most likely because the algae had allocated much of their resources to the  
21 215 earlier anomalous reproductive event. Therefore, only ca. 800 fertile apexes were harvested, and 146  
22 216 clay discs were seeded. The receptacle release efficiency ( $n = 30$ ) and germling length after one month  
23 217 of culture ( $n = 60$ ) were obtained as described above for the first cultivation.

24 218 In this case, despite the culture settings being the same as in the previous cultivation, the germlings  
25 219 showed slower growth. Therefore, to avoid a prolonged culture, which would have been highly  
26 220 demanding, the culture period was extended outdoors. In June 2019, after four weeks of culture, all  
27 221 the tiles were transported to the Miramare MPA, where they were affixed to a one-level plastic lantern  
28 222 net (diameter 55 cm) at 3 m depth and in close proximity to the previous outplanting (lantern net trial;  
29 223 Figure 3a). This kind of structure suspended in the water column is usually employed for the fattening  
30 224 and confinement of bivalves and sea cucumbers. In the present study, this device was used as an  
31 225 intermediate step between culturing and outplanting to mitigate the low culture efficiency.

32 226 After three months in the lantern net, the tiles were moved to the rocky bottom using the previously  
33 227 assembled modular frames. In the previous outplanting, the unprotected juveniles were heavily  
34 228 grazed, so all the tiles were protected by grazing deterrents (*P*).

35 229 To observe germling growth in the lantern net, germling length was measured on 30 random tiles at  
36 230 21 days (Time L1, where L refers to the lantern net trial), 30 days (Time L2) and 60 days (Time L3)  
37 231 after placement in the lantern net (Figure 3b). The juveniles' length was then measured on the  
38 232 outplanting date (Time L4) and at 30 days (Time L5) and 90 days (Time L6) after the outplanting  
39 233 (90, 120 and 180 days after deployment in the lantern net, respectively).

#### 56 234 **2.5 Statistical analyses**

57 235 The first cultivation tested the relationship between the percentage of germling survival at  $T_2$   
58 236 (response variable) and the germling density at  $T_1$  on the tagged tiles by means of a simple linear



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3 237 regression. To make the relationship linear, the values of the response variable were log-transformed.  
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5 238 Simple linear regression was also used to test the relationship between the germling density at  $T_2$  and  
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7 239 the germling density at  $T_1$  on the same tiles.  
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9 240 For the first outplanting, a linear mixed model (LMM) was created using the R package ‘lme4’ (Bates  
10 241 et al., 2015) to evaluate the effect of grazing deterrence on thallus length over time. The model  
11 242 included the *treatment* (two levels: *UP*, *P*) as a fixed effect and the sampling time as a random effect.  
12 243  $R^2$  values were calculated using the ‘r2glmm’ package (Jaeger, 2017). Pairwise Wilcoxon rank sum  
13 244 tests were used to compare the two treatments independently at each sampling time. All statistical  
14 245 analyses were performed using R 3.6.3 (R Core Team, 2020).  
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### 20 247 3. RESULTS

#### 21 248 3.1 First *ex situ* cultivation and outplanting

22 249 In culture, the release efficiency averaged  $9.5 \pm 1.4$  SE zygotes receptacle<sup>-1</sup>.  
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24 250 After two weeks ( $T_1$ ), the mean density was  $687 \pm 52$  SE germlings tile<sup>-1</sup> (corresponding to 54  
25 251 germlings cm<sup>-2</sup>  $\pm 4$ ), ranging from 36 to 2277 germlings tile<sup>-1</sup>. After four weeks ( $T_2$ ), the mean density  
26 252 was  $93 \pm 4$  SE germlings tile<sup>-1</sup> (corresponding to  $7.35$  germlings cm<sup>-2</sup>  $\pm 0.32$ ), ranging from 13 to 239  
27 253 germlings tile<sup>-1</sup>.  
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29 254 The mean percentage of germling survival between  $T_1$  and  $T_2$  was  $15.7\% \pm 1.4$  SE. A negative  
30 255 relationship was found between the survival percentage at  $T_2$  and the germling density at  $T_1$  (linear  
31 256 regressor  $\beta = -0.001$ ;  $p < 0.001$ ,  $R^2 = 0.43$ ), suggesting a density-dependent effect (Figure 4a).  
32 257 Nonetheless, the tiles with higher initial densities were also those with the highest final densities  
33 258 (Figure 4b): there was a positive relationship between germling density at  $T_2$  and germling density at  
34 259  $T_1$  (linear regressor  $\beta = 0.094$ ;  $p < 0.001$ ,  $R^2 = 0.63$ ).  
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36 260 At  $T_2$ , the germlings were  $1.72$  mm  $\pm 0.01$  SE long, with a maximum of 3.45 mm length (Figure 5).  
37 261 Two weeks after outplanting (Time G1), the mean germling length was  $0.8$  cm  $\pm 0.1$  SE in the *UP*  
38 262 treatment, while it was  $0.7$  cm  $\pm 0.1$  SE in the *P* treatment. After six months (Time G6), the juveniles  
39 263 were  $3.3$  cm  $\pm 0.3$  SE long in the *UP* treatment and  $6.2$  cm  $\pm 0.4$  SE long in the *P* treatment. The *P*  
40 264 treatment significantly affected juvenile length ( $p < 0.001$ ,  $R^2 = 0.25$ ; Table S2): starting at Time G3,  
41 265 the juvenile length in the *P* treatment was always greater than that in the *UP* treatment (Figure 6a,  
42 266 Figure S3c,d,e,f, Figure S6). The treatment also affected the density distribution of juvenile lengths;  
43 267 juvenile length was less evenly distributed on the *UP* tiles (Figure 6b).  
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45 268 After six months (Time G6), juveniles were present on 29% of the tiles in the *UP* treatment ( $1.2$   
46 269 individuals per tile  $\pm 0.07$  SE) and on 46% of the tiles in the *P* treatment ( $1.5 \pm 0.09$  SE) (Figure 7).  
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3 270 The visual census of *S. salpa* detected the presence of approximately 1,600 specimens inside the 30-  
4 271 ha “no-take” area of the MPA.

### 6 272 **3.2 Second *ex situ* cultivation and outplanting**

8 273 In culture, the release efficiency averaged  $0.7 \pm 0.1$  SE zygotes receptacle<sup>-1</sup>, and the mean germling  
9 274 length after four weeks of culture was  $0.4 \text{ mm} \pm 0.03$  SE.

11 275 In the field, the germlings in the lantern net were  $1.4 \text{ cm} \pm 0.1$  SE long after one month (Time L2)  
13 276 and  $1.6 \text{ cm} \pm 0.11$  SE long after two months (Time L3); in the third month (Time L4), a decrease in  
15 277 length due to fish grazing was detected ( $1.2 \text{ cm} \pm 0.08$  SE). In fact, signs of fish bites were evident  
17 278 on many juveniles (Figure 8). Once the juveniles moved to the rocky bottom and obtained protection  
19 279 (the *P* condition), they grew to  $2.9 \text{ cm} \pm 0.3$  SE long at Time L6 (Figure 9).

21 280 The juveniles in the second experiment, unlike those in the first experiment, did not become fertile in  
22 281 the following spring (Figure S7).

## 25 283 **4. DISCUSSION**

27 284 The restoration of key species and habitats is promoted internationally to reverse the shift from highly  
28 285 diverse ecosystems to less diverse ones and thus to re-establish the services that ecosystems provide  
30 286 (Biodiversity Strategy to 2020 – European Commission, 2011; Biodiversity Strategy to 2030 –  
32 287 European Commission, 2020; UN Decade on Ecosystem Restoration – General Assembly of the  
34 288 United Nations, 2019).

36 289 Among the factors that can affect the restoration process, climate change, whose effects on target  
37 290 species are poorly known, can impair restoration success in unpredictable ways. Although various  
38 291 studies support the hypothesis that temperature plays a key role in the gametogenesis and gamete  
40 292 release of Fucales (e.g. Norton, 1981; Bacon & Vadas, 1991; Pang et al., 2009; Kraufvelin et al.,  
42 293 2012; Falace et al., 2018), information on its role in these processes is insufficient (de Bettignies,  
44 294 Wernberg & Gurgel, 2018). In addition, although the anticipation of spring phenology due to global  
46 295 warming is on average stronger for marine species than for terrestrial species (as reviewed in  
47 296 Poloczanska et al., 2013 and in de Bettignies, Wernberg & Gurgel, 2018), this is the first study in  
49 297 which the effects of a reproductive shift in response to a sea thermal anomaly are assessed for seaweed  
51 298 in a restoration context.

53 299 In this study, the second culture was less efficient than the first culture in terms of zygote release (ten  
54 300 times lower) and germling growth (four times shorter), despite both cultures being carried out under  
56 301 the same laboratory conditions. The natural variability in propagule release among furoid species is  
58 302 well known (Gunnill, 1980; Reed, 1990; Vadas, Johnson & Norton, 1992); therefore, differences in  
59 303 the release efficiency could be expected. In this case, however, the MHW likely exacerbated this

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3 304 natural variability and affected germling growth as well. As it was already fertile in February, *T.*  
4 305 *barbata* was most likely at the end of its reproductive cycle in May, as also evidenced by the poor  
5 306 development of branches and receptacles compared to the usual spring phenology in the Gulf of  
6 307 Trieste (Falace & Zanelli, 2006).

10 308 This result highlights how reproductive stochasticity may represent a major threat to the  
11 309 implementation of large-scale restoration actions in the future, as thermal anomalies are being  
12 310 recorded with increasing frequency throughout the ocean and are likely to shape future marine  
13 311 ecosystems (Frölicher & Laufkötter, 2018; Pastor, Valiente & Palau, 2018; Wernberg et al., 2019).  
14 312 Early-warning networks regarding extreme climatic events might allow restoration practitioners to  
15 313 monitor the phenological responses of *Cystoseira*, collect prematurely fertile receptacles in order to  
16 314 cultivate *Cystoseira* under controlled mesocosm conditions, or select lineages with higher plasticity  
17 315 to thermal extremes, increasing the chance for successful restoration actions (Bevilacqua et al., 2019).  
18 316 After one year (March 2020), the taller thalli (approximately 14-17 cm) grown in the first outplanting  
19 317 were starting to develop receptacles. This development did not occur in plants in the second  
20 318 outplanting, likely because after six months, the juveniles in the first outplanting (the *P* treatment)  
21 319 were twice as long as those in the second outplanting ( $6.2 \text{ cm} \pm 0.4 \text{ SE}$  versus  $2.9 \text{ cm} \pm 0.3 \text{ SE}$ ). The  
22 320 difference in length likely occurred because the juveniles in the first outplanting benefited from  
23 321 favourable seasonal conditions (e.g. the extended photoperiod in the summertime) for a longer period.  
24 322 This outcome suggests that, in a restoration context, altered life cycles and delayed growth could also  
25 323 hinder spill-over dynamics in the restored plantlets as adults and slow the re-establishment of self-  
26 324 sustaining populations.

29 325 Despite the anomalous receptacle development, in the first culture, the cultivation protocol resulted  
30 326 in a higher final germling length than in a previous study on the same species. After one month, the  
31 327 germling length was  $1.72 \text{ mm} \pm 0.01$ , which is six times longer than that reported by Verdura et al.  
32 328 (2018) (i.e. 0.2 – 0.4 mm), and germlings in that study reached 5 mm after six months. Our results  
33 329 are comparable to those obtained with *C. amentacea* in Falace et al. (2018) (i.e.  $1.38 \text{ mm} \pm 0.13 \text{ SE}$   
34 330 after a three-week culture).

35 331 The germling density averaged  $54 \text{ germlings cm}^{-2} \pm 4 \text{ SE}$  after two weeks of culture, a lower value  
36 332 than in Falace et al. (2018) for *C. amentacea* ( $160 \text{ germlings cm}^{-2} \pm 47$ ). However, the mean density  
37 333 value obtained after a month ( $7.35 \text{ germlings cm}^{-2} \pm 0.32$ ) was similar to the value reported for  
38 334 *Treptacantha elegans* by Medrano et al. (2020a) after two months of culture (i.e.  $10.14 \text{ germlings}$   
39 335  $\text{cm}^{-2} \pm 5.30$ ).

40 336 As expected, the germling survival rate in culture (average 16% after four weeks) was higher than  
41 337 that reported in natural stands (e.g. Gunnill, 1980; Schiel, 1988; Lamote & Johnson, 2008; Capdevila

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3 338 et al., 2018). For example, Wright, Williams & Dethier (2004) reported that in *Fucus gardneri*, less  
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5 339 than 0.0004% of embryos survived to become visible recruits, while Chapman (1995) observed that  
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7 340 in *F. distichus*, only 0.4-12% of settlers became visible recruits.

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9 341 The negative relationship between survival rate and germling density can be explained by the  
10 342 progressive decline in the density of a population of growing plants (i.e. the “self-thinning” process;  
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12 343 e.g. Black, 1974; Santelices et al., 1980; Dean, Thies & Lagos, 1989; Ang & De Wreede, 1992; Creed,  
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14 344 Norton & Kain, 1997; Steen & Scrosati, 2004).

15 345 Controlling for grazers improved the performance of the outplantings. After six months, the number  
16  
17 346 of juveniles per tile ranged between one and two in both treatments, but in the *P* treatment, the  
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19 347 percentage of tiles with at least one juvenile was almost twofold that in the *UP* treatment. In addition,  
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21 348 the juveniles were on average two times larger in the *P* treatment, and their length distribution  
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23 349 resembled that of a natural population. According to Verdura et al. (2018), a population with a well-  
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25 350 represented size distribution is indicative of a successful restoration effort.

26 351 *Sarpa salpa*, which is highly abundant inside the MPA, was most likely the main grazer on the  
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28 352 outplanted juveniles; schools were often observed around the experimental plots during monitoring  
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30 353 by scuba divers and on camera, and the plantlets showed evident signs of fish bites. Conversely,  
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32 354 grazing by sea urchins (one of the major drivers of the loss of *Cystoseira* stands; e.g. Verlaque, 1984;  
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34 355 Hereu, 2004; Nikolić et al., 2013) does not need to be considered in the present study because sea  
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36 356 urchins were never observed on the tiles and are scarce in the MPA. The impact of mesograzers (e.g.  
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38 357 molluscs, crustaceans) as observed in the videos was also likely negligible, most likely because the  
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40 358 germlings were outplanted having attained “refuge” size; this finding highlights the importance of  
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42 359 maximizing germling growth in nurseries.

43 360 The grazing deterrents, in addition to successfully protecting the outplanted juveniles, reduced the  
44  
45 361 risks of detachment and loss related to hydrodynamism compared to large cages affixed with epoxy.  
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47 362 However, they require the same periodical maintenance; thus, their deployment in large-scale  
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49 363 interventions would be unsustainable. A possible, though controversial, option that has been  
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51 364 suggested for achieving long-term restoration success in sites with high grazing pressure is the  
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53 365 restoration of trophic interactions or herbivore culling (e.g. Filbee-Dexter & Scheibling, 2014; Gianni  
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55 366 et al., 2018; Carlsson & Christie, 2019; Medrano et al., 2020a; Eger et al., 2020).

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57 367 The modular frames proved to be a suitable approach for outplanting subtidal *Cystoseira* species.  
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59 368 Screwing the tiles onto the preassembled modular structures decreases the required time, costs and  
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369 personnel compared with other methods, thus showing great upscaling potential. In fact, a) tile  
370 screwing can be rapidly performed on land (three hours and four people for 400 tiles); b) scuba divers  
371 can easily and rapidly deploy a large number of tiles, reducing the stress on germlings (two hours and

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372 two people, considering both the drilling activities before deployment and fastening the module to  
373 the rock); c) the modular frames can be arranged to mimic the different patterns of *Cystoseira*  
374 distribution in natural stands; and d) not using epoxy putty to fix each tile to the rocky substrate  
375 minimizes the aesthetic and environmental impacts of the outplanting and guarantees the stability of  
376 the outplants in the first hours after deployment, before putty hardening (De La Fuente et al., 2019).  
377 The lantern net allowed us to reduce the prolonged cultivation duration that would have been required  
378 for the germlings to reach refuge size in the second experiment because of the slow germling growth.  
379 The suspended *in situ* cultivation also seemed to restrict the presence of grazers, which can typically  
380 be found on vegetated rocky bottoms. However, fish bites started to be detected when the juveniles  
381 were approximately 1.5-2 cm long (Time L4) as in the *UP* treatment on the sea bottom (Time G3);  
382 this result suggests that juveniles start to become attractive to *S. salpa* when they reach approximately  
383 1.5-2 cm in length.

384 Large-scale restoration must be cost-effective and requires the production of large quantities of  
385 germlings; however, production is hampered by the availability of facilities (e.g. the dimension and  
386 number of environmentally controlled rooms, the number of aquaria, labour), which requires many  
387 consecutive cultivation cycles to be performed. However, the number of possible cultivation cycles  
388 is limited by the narrow reproductive period of the majority of furoids (e.g. two months in the  
389 Northern Adriatic Sea for *T. barbata* – Falace & Bressan, 2006). Coupling the culture in the  
390 mesocosms to a transitional step in the field (e.g. a floating lantern net) before outplanting on rocky  
391 bottoms in a three-step process allows the advantages of both *ex situ* and *in situ* techniques to be  
392 combined. The recruitment enhancement achieved through optimized culture conditions in  
393 mesocosms would a) reduce the impact of unpredictable natural events, b) increase settlement  
394 success, c) maximize the survival of the most critical early developmental stages, and d) allow us to  
395 produce more and larger germlings in a shorter time. Suspended *in situ* cultivation, which reduces the  
396 amount of time that the germlings stay in the mesocosms, is beneficial because: a) it prevents the risk  
397 of culture contamination by bacteria and epiphytes, which often occurs in prolonged laboratory  
398 cultures, and b) it is cost-effective; with a low investment, it avoids time-consuming and costly  
399 maintenance in the laboratory.

400 Currently, comparisons of the restoration costs between the proposed methodology and those reported  
401 in other studies (i.e. Verdura et al., 2018; Medrano et al., 2020a) are almost impossible, considering  
402 that the restoration parameters (such as the hourly cost per person, depreciation of the costs incurred  
403 in building the nurseries, culture yield) have not been standardized. Meaningful comparative cost  
404 analyses would be feasible only in an aquaculture context, with optimized protocols and with



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3 405 specialized semi-industrial infrastructure, and are not feasible while this methodology is still in an  
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5 406 experimental phase.

6 407 In conclusion, it is evident that the restoration of fucoids is still in its infancy. It is necessary to  
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8 408 proceed with experimental trials to test culturing and outplanting methods tailored to the target  
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10 409 species and habitats before planning large-scale interventions. In addition, a better understanding of  
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12 410 the relationships of the early fucoid life stages to disturbance/variation in the physical environment  
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14 411 as well as to climate change should be achieved, and these relationships should be considered within  
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16 412 the context of effective large-scale restoration efforts.

17 413

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41 764

## 51 765 **FIGURE LEGENDS**

52 766 Figure 1 – *Treptacantha barbata* a) in February 2019, in the donor site with the winter *habitus*, and  
53 767 b) as it usually appears during the springtime maximum development in the Gulf of Trieste (never  
54 768 observed during this study) (credits: D. Curiel). c) Receptacles developing on adventitious branches  
55 769 (i.e. first *ex situ* cultivation). Receptacles developing on primary branches: d) small (as collected for  
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3 770 the first and second *ex situ* cultivation) and e, f, g, h) fully developed (never observed during this  
4 study).

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8 773 Figure 2 – The outplanting modular structure: a) “Unprotected” (*UP*) and b) “Protected” (*P*). The  
9 grazing deterrents were formed by c) raising the flexible metallic mesh strips around the tile, as petals  
10 774 of a flower. Each modular structure had a surface of 0.5 m<sup>2</sup> (0.5 m x 1 m).

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15 777 Figure 3 – Suspended *in situ* cultivation in the second restoration trial. a) The lantern net and b) a  
16 detail of the tiles fixed to the bottom of the floating structure.

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20 780 Figure 4 – Germling survival and density in the first culture. a) Relationship between germling %  
21 survival at T<sub>2</sub> and the germling density at T<sub>1</sub> (No. of germlings/tile). b) Relationship between germling  
22 781 density at T<sub>2</sub> and the germling density at T<sub>1</sub>, expressed in terms of No. of germlings per tile. The red  
23 782 lines represent the calculated regression trend.

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29 785 Figure 5 – Variability of germling length (mm) after four weeks of culture in the first cultivation (T<sub>2</sub>).  
30 The solid line within the box represents the median.

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34 788 Figure 6 – Growth of the juveniles in the first outplanting. a) Boxplots of the thallus lengths (cm) in  
35 the two treatments, “Unprotected” (*UP*) and “Protected” (*P*). b) Size-class distribution of the restored  
36 789 population over time. The fish icon represents the sampling time at which fish grazing on the juveniles  
37 790 started to be detected.

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43 793 Figure 7 – Percent tiles with juveniles after six months in the field (Time G6) in the two treatments  
44 (“Unprotected” – *UP*, “Protected” – *P*) from the first outplanting.

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48 796 Figure 8 – Length of juveniles from the first (“Unprotected” – *UP*, “Protected” – *P*) and the second  
49 (“Lantern net” – *L*) outplanting. The fish icon represents the sampling time at which signs of fish  
50 797 grazing on the juveniles started to be detected.

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55 800 Figure 9 – Juvenile length (cm) when the tiles were in the lantern net (blue) and on the sea bottom  
56 (brown). Juveniles were moved to the sea bottom at Time L4. The fish icon represents the sampling  
57 801 time at which signs of fish grazing on the juveniles started to be detected.

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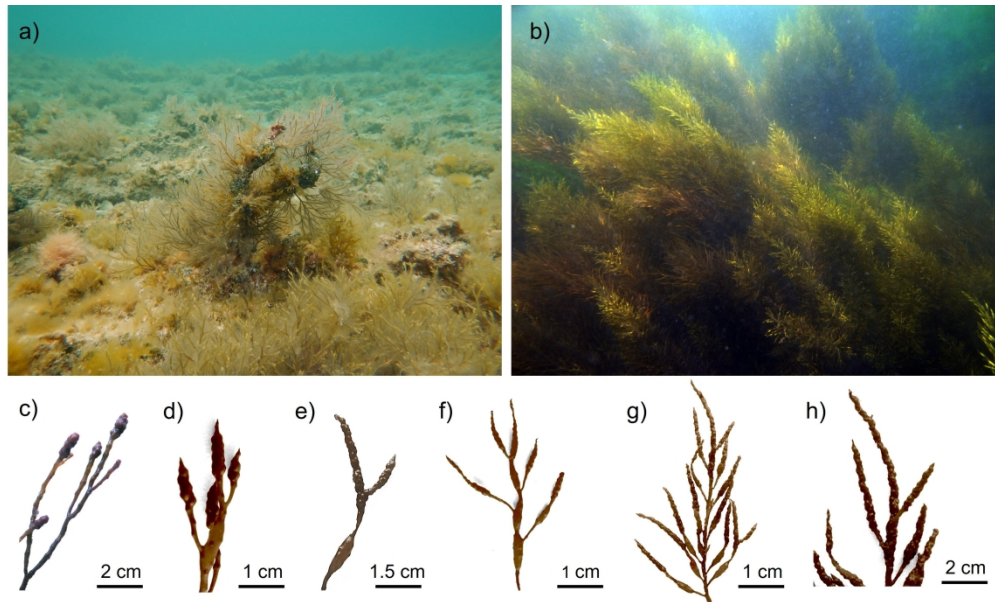


Figure 1 – *Treptacantha barbata* a) in February 2019, in the donor site with the winter habitus, and b) as it usually appears during the springtime maximum development in the Gulf of Trieste (never observed during this study) (credits: D. Curiel). c) Receptacles developing on adventitious branches (i.e. first *ex situ* cultivation). Receptacles developing on primary branches: d) small (as collected for the first and second *ex situ* cultivation) and e, f, g, h) fully developed (never observed during this study).

337x204mm (300 x 300 DPI)



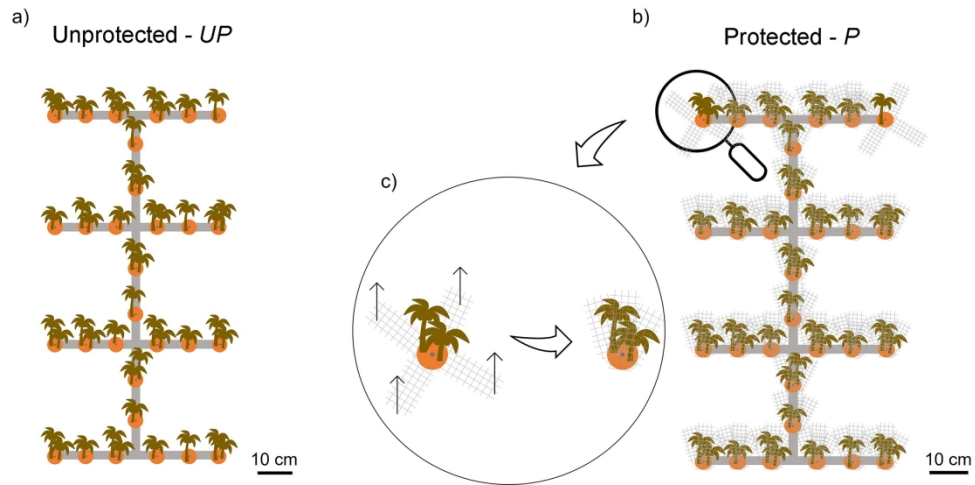


Figure 2 – The outplanting modular structure: a) “Unprotected” (UP) and b) “Protected” (P). The grazing deterrents were formed by c) raising the flexible metallic mesh strips around the tile, as petals of a flower. Each modular structure had a surface of 0.5 m<sup>2</sup> (0.5 m x 1 m).

121x59mm (600 x 600 DPI)

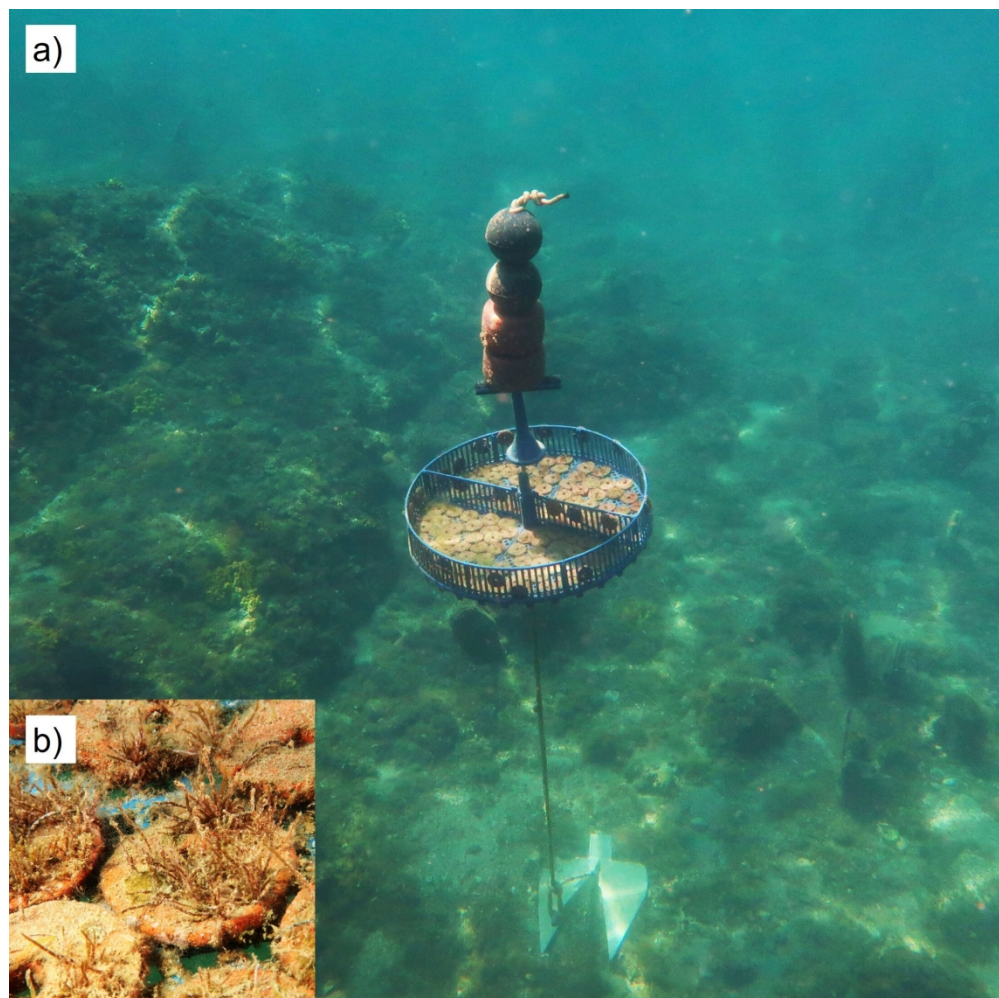


Figure 3 – Suspended *in situ* cultivation in the second restoration trial. a) The lantern net and b) a detail of the tiles fixed to the bottom of the floating structure.

187x186mm (300 x 300 DPI)

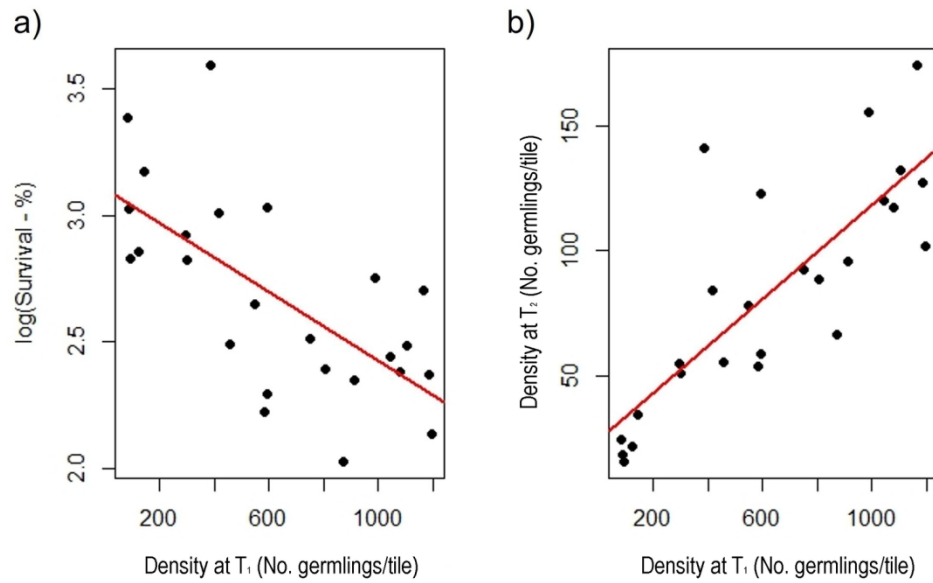


Figure 4 – Germling survival and density in the first culture. a) Relationship between germling % survival at T<sub>2</sub> and the germling density at T<sub>1</sub> (No. of germlings/tile). b) Relationship between germling density at T<sub>2</sub> and the germling density at T<sub>1</sub>, expressed in terms of no. of germlings per tile. The red lines represent the calculated regression trend.

74x46mm (600 x 600 DPI)

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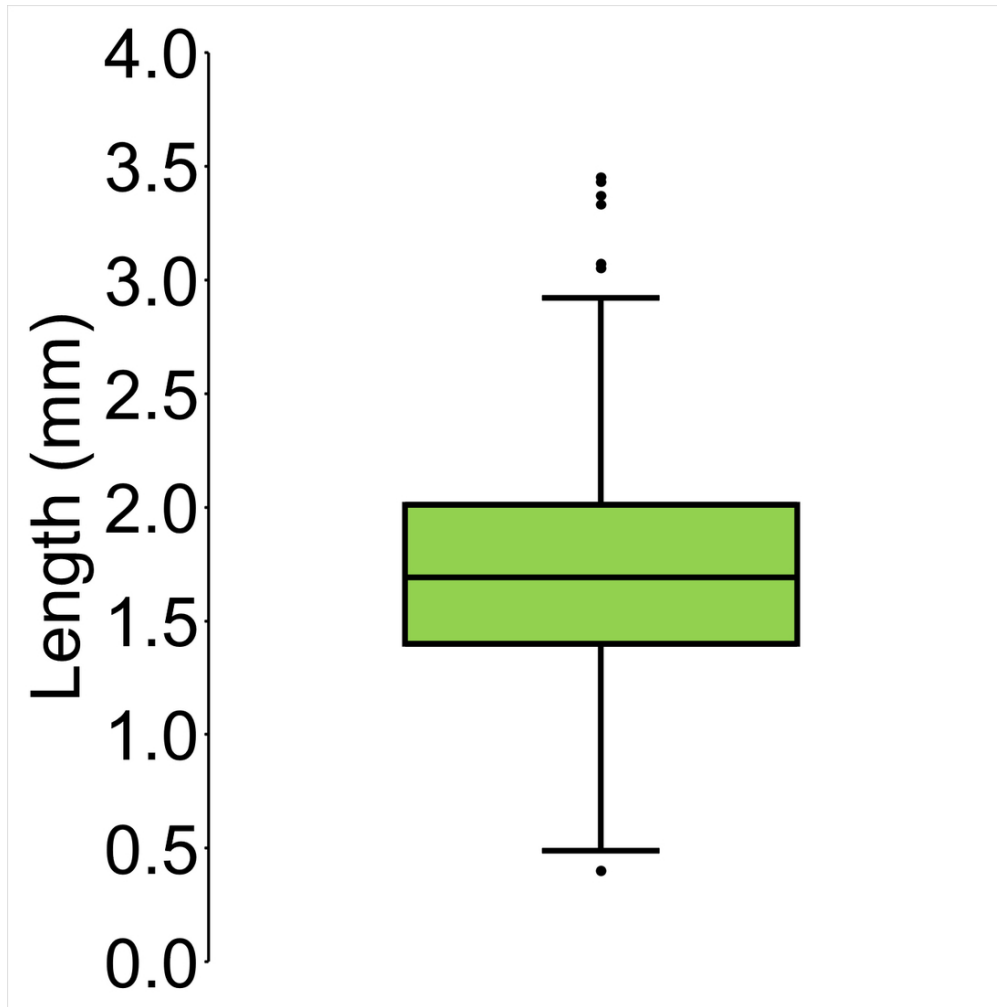


Figure 5 – Variability of germling length (mm) after four weeks of culture in the first cultivation (T<sub>2</sub>). The solid line within the box represents the median.

46x46mm (600 x 600 DPI)

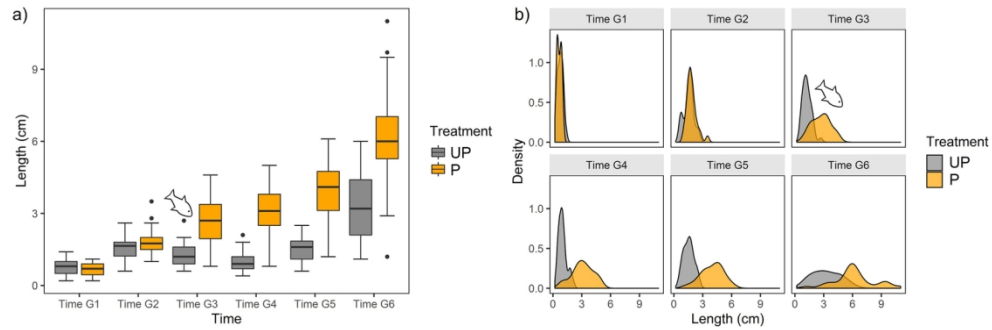


Figure 6 – Growth of the juveniles in the first outplanting. a) Boxplots of the thallus lengths (cm) in the two treatments, “Unprotected” (*UP*) and “Protected” (*P*). b) Size-class distribution of the restored population over time. The fish icon represents the sampling time at which fish grazing on the juveniles started to be detected.

138x46mm (300 x 300 DPI)



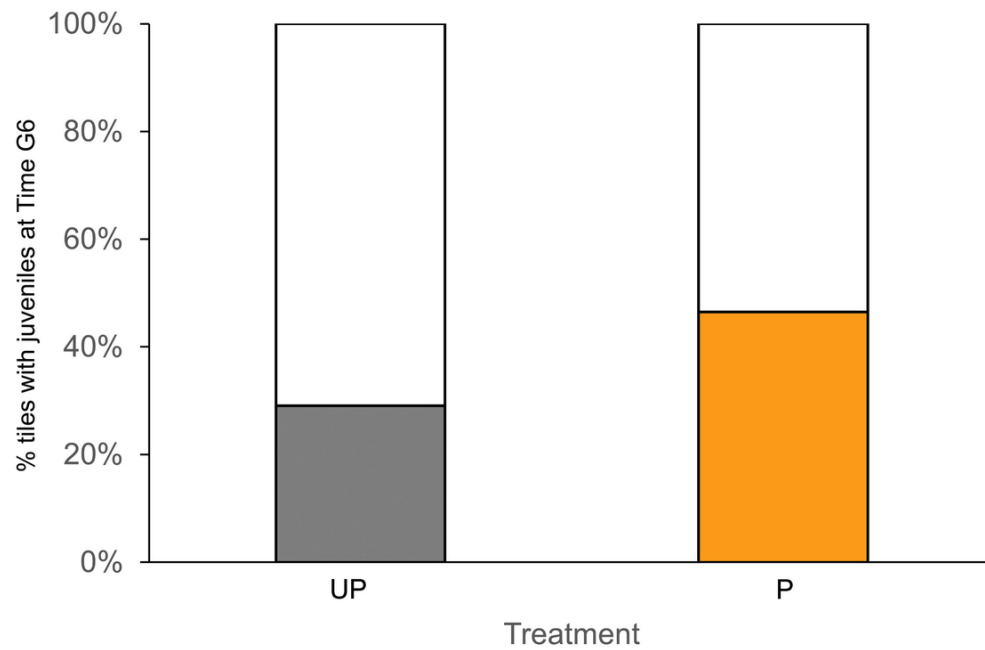


Figure 7 – Percent tiles with juveniles after six months in the field (Time G6) in the two treatments ("Unprotected" – *UP*, "Protected" – *P*) from the first outplanting.

94x61mm (300 x 300 DPI)

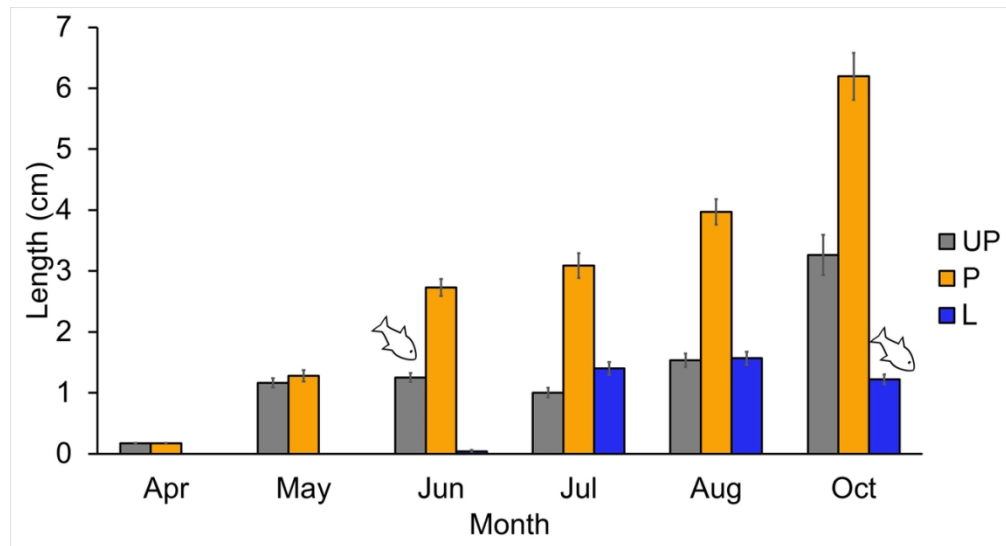


Figure 8 – Length of juveniles from the first (“Unprotected” – *UP*, “Protected” – *P*) and the second (“Lantern net” – *L*) outplanting. The fish icon represents the sampling time at which signs of fish grazing on the juveniles started to be detected.

112x61mm (300 x 300 DPI)

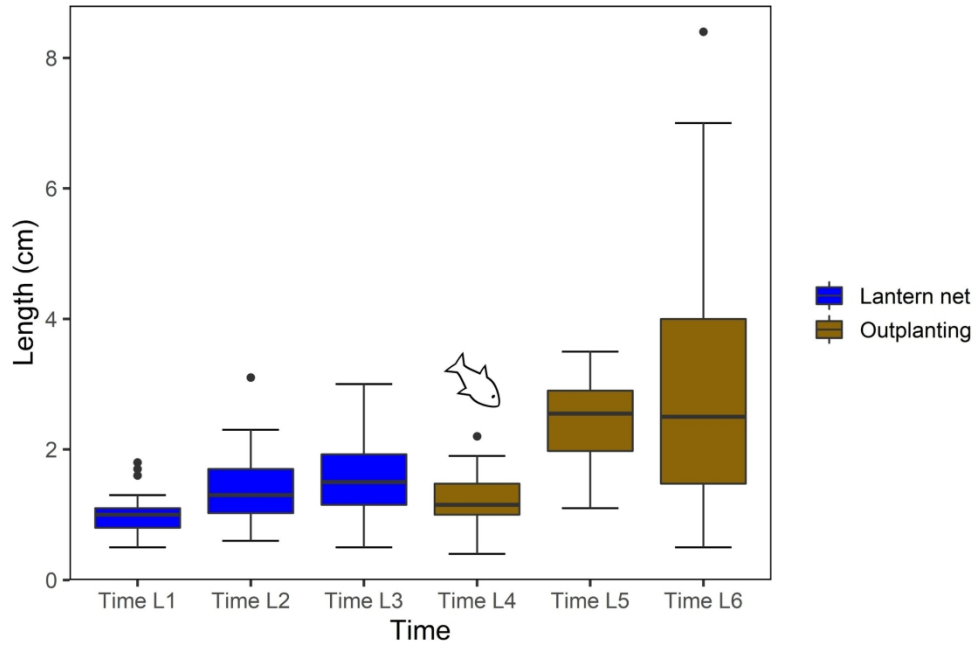


Figure 9 – Juvenile length (cm) when the tiles were in the lantern net (blue) and on the sea bottom (brown). Juveniles were moved to the sea bottom at Time L4. The fish icon represents the sampling time at which signs of fish grazing on the juveniles started to be detected.

91x61mm (600 x 600 DPI)