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**Stranded seaweeds (*Gongolaria barbata*): an opportunity for macroalgal forest restoration**

Running head: New protocol for *Gongolaria barbata* restoration

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## 24    **Abstract**

25    Macroalgal forests play a crucial ecological role, providing important ecosystem services, but are also  
26    among the most vulnerable marine habitats. In the Mediterranean Sea the forests of *Cystoseira sensu*  
27    *lato* (*s.l.*) are undergoing a drastic decline due to the presence of multiple stressors and, among these  
28    species, *Gongolaria barbata* is one of the most threatened. Despite the various attempts to restore  
29    these macroalgal forests, the success of the interventions is limited by the availability of fertile apices  
30    to promote zygotes release and the embryo development for subsequent replacement *in situ*. Here we  
31    propose a new approach based on the use of *G. barbata* stranded on the beach, for the restoration of  
32    these marine habitats. We developed a protocol based on the collection of stranded macroalgae to  
33    collect their fertile apices and produce healthy zygotes and embryos, whose recruits can be then  
34    returned at sea (through *ad hoc* hard substrates). We show that 3 months of incubation in mesocosms  
35    allow the recruits to reach an average length of 1 – 2 mm with an average density of 50 – 80 recruits  
36    per tile. We demonstrate that these recruits can survive and grow vigorously both in mesocosms, and  
37    after outplanted at sea. The protocol presented here has the double advantage: a) obtaining recruits  
38    without impacting the natural populations, and b) providing to a second life to macroalgal fragments  
39    (through ecological restoration) that would be otherwise lost.

40

41    **Key-words:** macroalgal restoration; *ex situ* cultivation; *Gongolaria barbata*; *Cystoseira s.l.*;  
42    Mediterranean Sea.

43

## 44    **Implications for Practice and Technique**

- 45    • A new protocol is described for the restoration of the seaweeds (*Gongolaria barbata*), having the  
46       advantage of obtaining recruits from stranded individuals without any damage to the natural  
47       populations.
- 48    • The protocol exploits stranded fertile adults which produce healthy recruits, thus representing a  
49       potential source of fertile apices. Stranded adults could be thus used in restoration actions instead  
50       of being lost.
- 51    • The results obtained applying this protocol, in terms of new recruits survival % and growth, are  
52       similar to those obtained with other approaches known for this species.

53

## 54    **Introduction**

55    *Cystoseira sensu lato* (*s.l.*) (Phaeophyceae, Fucales) species, including the genera *Ericaria*,  
56    *Gongolaria* and *Cystoseira*, are foundation species playing a key role as habitat-formers on the  
57    Mediterranean rocky bottoms (Orlando-Bonaca et al. 2021). They ensure high primary production,  
58    food sustaining grazers, and nurseries for a large variety of species (Chiarore et al. 2019; Bianchelli  
59    & Danovaro 2020). Moreover, they can support high biodiversity levels, due to their three-  
60    dimensional structure providing a relevant “ecological volume” and contributing to ecosystem  
61    services (Thibaut et al. 2016).

62       In the last decades, the decline of *Cystoseira s.l.* populations was documented along many  
63    Mediterranean coasts (Thibaut et al. 2015; Iveša et al. 2016), due to multiple natural and  
64    anthropogenic stressors (e.g., habitat destruction, pollution, overgrazing, alien species; Sales &  
65    Ballesteros 2009; Marletta & Lombardo 2020), exacerbated by the effects of climate change,  
66    including marine heatwaves (Lejeusne et al. 2010; Bevilacqua et al. 2019; Garrabou et al. 2022).

67       A dramatic loss of furoid species was reported also in the Adriatic Sea (Rindi et al. 2020),  
68    with their replacement with less-structured turf-forming algae, preventing in turn canopy-forming-

69 species recruitment (Orlando-Bonaca et al. 2021). This phenomenon was widely documented along  
70 the Conero Riviera (North-Eastern Adriatic Sea) for four furoid species (*Fucus virsoides*, *Cystoseira*  
71 *foeniculacea*, *Cystoseira humilis*, *Sargassum acinarium*). The main driver of this forest loss is the  
72 human impact on coastlines, increasing sediment resuspension, high nutrient concentrations and loads  
73 (Rindi et al. 2020). As a result, only *Cystoseira compressa* and *Gongolaria barbata* are still present,  
74 even though the latter species is in regression, due to their greater tolerance to environmental stress  
75 (Orlando-Bonaca et al. 2022).

76 In the last years, the possibility of restoring these macroalgal forests (e.g., *G. barbata*) was  
77 explored in the Adriatic Sea (Savonitto et al. 2021; Orlando-Bonaca et al. 2022). The restoration of  
78 fucalean forests is carried out either using *in situ* and *ex situ* (cultivated in mesocosms) recruitment  
79 approaches or using hybrid methods (combining cultivation in mesocosm and culture in the natural  
80 environment, also coupled with passive restoration measures (Verdura et al. 2018; Orlando-Bonaca  
81 et al. 2022; Bianchelli et al. 2023a; 2023b).

82 Nevertheless, climate change and thermal anomalies might jeopardise conservation and  
83 restoration efforts, compromising the future viability of brown algae of *Cystoseira s.l.* species in the  
84 Mediterranean (Iveša 2019; Orlando-Bonaca et al. 2021). Recent studies highlighted that variability  
85 in the magnitude of marine heat waves can lead to local extinctions of already fragmented populations  
86 in the Mediterranean Sea (Verdura et al. 2021). Yet, the increasing storm frequency and intensity  
87 promote the detaching of macroalgae from substrates and the stranding on the beach (Pisano et al.  
88 2020). The Adriatic Sea is particularly exposed to storm surges due to the scirocco winds, which  
89 produce the largest wave heights (Pomaro et al. 2017), thus fostering the fragmentation or local  
90 extinction of Adriatic populations.

91 Moreover, seawater warming can alter physiology and phenology of *G. barbata*, impairing  
92 reproductive timing and recruitment (Eggert 2012; Bevilacqua et al. 2019). Altogether, these factors  
93 might have a negative synergistic effect (Bevilacqua et al. 2019; Garrabou et al. 2022), compromising

*G. barbata* zygote availability, and reducing its reproductive potential (Savonitto et al. 2021). Therefore, the interactions of these factors might become limiting for the success of a restoration intervention (Verdura et al. 2018).

Recently, the use of plants fragments naturally detached from the meadows after storms has been suggested as a valid non-destructive method for seagrass restoration, not only to limit the impact on natural meadows, but also for the low probability of flowering of Mediterranean seagrass species (Ferretto et al. 2021). However, to our best knowledge, the implementation of detached fertile fragments from furoid species in restoration interventions has never been reported.

Here we explored the potential use of *G. barbata* stranded along the shores after a storm, for the restoration of this species. The aim of this study was to assess the efficacy of an approach based on the collection of fertile stranded specimens of *G. barbata*, otherwise lost from the marine habitats, to generate vital and healthy offspring than can promote the recovery of this species.

## **Methods**

### ***Sample collection***

In April 2023, fertile thalli of *Gongolaria barbata* were found, stranded along the Sassonia beach and two locations of Fano shoreline (Lido 1 and Lido 2) (North-Eastern Adriatic Sea; Figure 1A-B). Fano is a touristic city hosting a marina and breakwaves along the shoreline. The sampling sites are subjected to rough sea weather conditions and storms usually occur from autumn to spring (Rindi et al. 2020). The presence of *G. barbata* along the Fano shore was never reported before. However, during a previous survey in the area, we detected scattered individuals both on the artificial breakwaters and on *Sabellaria* reefs in the area.

For each location, 2 thalli were collected and their sizes (length of the thalli, receptacles and aerocysts) were measured. Subsequently the thalli were brought to the laboratory and stored in 40 L tanks at the Fano Marine Centre. Three receptacles from each thallus were taken and observed at the

119 stereomicroscope. Longitudinal and transversal sections were made through a razor blade to verify  
120 their fertility and evaluate the receptacles and gametes morphometric variables (length of the  
121 receptacles, antheridia and oogonia). The reproductive period of *G. barbata* in the Adriatic Sea  
122 generally occurs mostly in spring and early summer (Rindi et al. 2023). The fertility was then checked  
123 and assessed by counting the number of released zygotes in 3 receptacles taken from the different  
124 thalli, positioned above slides and placed within a petri with filtered seawater.

125

### 126 ***G. barbata maintenance and reproduction***

127 The macroalgae were transferred to the aquarium facility at the Polytechnic University of Marche  
128 (Ancona city) and acclimatized before their cultivation in mesocosms, by slow mixing the seawater  
129 used for the re-hydration and transport and the seawater from the tanks. Three mesocosms were used,  
130 one for each sampling location. For determining the efficiency of the reproductive thalli in producing  
131 healthy offspring, 4 clay tiles were located in each tank under the 2 fertile specimens (i.e., two  
132 individuals for each sampling location) to allow the zygotes and embryos to settle. The tiles were 7  
133 cm in diameter and 1.5 cm in thickness, with a hole at the center, to be later mounted on a steel  
134 structure to be out planted at sea.

135 To optimize the maintenance of algae, we used the LSS (Life Support System) consisting in  
136 3 x 50-L mesocosms, a reserve in which there are 3 socks of 100  $\mu\text{m}$  for mechanical filtration,  
137 immersed razor clams for biological filtration. Fluorescent lamps produced 260-nm ( $\lambda$ ) UV-C rays,  
138 sterilizing the water, damaging nucleic acids and preventing microbes' proliferation. A Teco TK 500  
139 cooler was used for maintaining the temperature. The light intensity was generated by 2 LED lamps  
140 (SilverMoon Marine 10 thousand Kelvin and SilverMoon Universal 6.5 thousand Kelvin) 40 cm away  
141 from the water head. Irradiance was measured with Photometer of the apogee Model MQ-500. Light  
142 intensity and photoperiod were selected to reflect typical seasonal conditions during the reproductive  
143 phase of *G. barbata* (Savonitto et al. 2021). The photoperiod was set to a 15:9 h light : dark cycle,  
144 and light intensities was 80-100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . The cultivation medium was Von Stosch's

145 enriched filtered seawater renewed every 2 weeks, with addition of germanium dioxide to prevent  
146 microalgal proliferation. Temperature, salinity, pH and light intensity were monitored over the  
147 duration of the experiments. Furthermore, for routine maintenance of the system, water loading and  
148 unloading, lights, movement pumps, cooler, any water leaks at the pipe joints were checked. To assure  
149 the sterilization of the system, once a week the socks were washed, tubs were siphoned to remove  
150 organic debris and 10% of the seawater was exchanged every week.

151 Two weeks after, when the recruits were visible at the stereomicroscope, their growth was  
152 assessed in term of height and density (as number of recruits per tile) for 3 months. Recruits height  
153 was measured on 15 recruits and the density as number of recruits on 4 standard areas of each tile.  
154 Data were collected using a stereomicroscope (magnification 6.4x), an Olympus TG-6 camera and  
155 were then analyzed with the software ImageJ.

156

### 157 ***Outplanting and monitoring recruits in the field'***

158 After 3 months, the tiles were transported to La Vela location, Portonovo (43.55° N – 13.60°E, ca-  
159 58-km distant from Fano), where the historical presence of *G. barbata* was previously documented  
160 (Perkol-Finkel & Airoidi 2010). *G. barbata* current occurrence along the Conero Riviera has been  
161 recently documented (Orfanidis et al. 2021). Portonovo is nearby two Sites of Community Importance  
162 (SCI IT5230005 “Coast between Ancona and Portonovo” and IT5320006 “Portonovo and calcareous  
163 cliff”; Bianchelli et al. 2023a).

164 The tiles were mounted on 3 45-cm-long steel structures and fixed to the sea bottom by four  
165 screws, at about 2 m of water depth. The tiles from each of the 3 locations were placed on a single  
166 structure, about 2 cm distant one from each other within each structure. The 3 structures were  
167 approximately 5 m apart from each other.



168           The tiles were monitored for 4 weeks after outplanting and photographed through an Olympus  
169 TG-6 camera. To estimate the recruits density and survival % for each tile, 3 photos were analyzed.  
170 The height of recruits was measured on 5 specimens per 3 tiles ( $n = 15$ ) using a ruler. The survival %  
171 of recruits was calculated as  $n. \text{ recruits at } 4^{\text{th}} \text{ week} / n. \text{ recruits at the beginning of the outplant} \times 100$ .

172

### 173 *Statistical analyses*

174 Due to the non-independence among the 3 mesocosms, data on the recruits height and density from  
175 the 3 locations (Lido 1, Lido 2 and Sassonia) in the lab phase were treated only with descriptive  
176 statistic. For data collected in the field on recruits height, density and survival %, two-way ANOVA  
177 was used to test for significant effect of the factors: “time” (fixed, 2 levels: first and fourth week,  
178 corresponding to the beginning and the end of the monitoring) and “location” (fixed, 3 levels: Lido  
179 1, Lido 2 and Sassonia). Assumptions of normality (Shapiro-Wilk) were previously checked. When  
180 significant effect of the 2 factors was observed, post-hoc analyses were carried out by Tukey test. All  
181 statistical analyses were performed through the software Jamovi 2.3 (Jamovi project, 2022).

182

## 183 **Results**

### 184 *Stranded macroalgal fragments*

185 The specimens stranded were 20 – 30 cm in length (Figure 2A). The receptacles, brought on terminal  
186 branchlets, were sickle-shaped, solitary or branched (Figure 2B-C). The single or two-in-chain  
187 aerocysts were located at the base of the branchlets (Figure 2B).

188           All the collected specimens were fertile. In longitudinal section, inside the receptacles the  
189 number of conceptacles were 10-15, in parallel rows (Figure 2D). In cross-section, dark and oval  
190 oogonia and pigmented and poorly-branched antheridia were, respectively, in groups on the floor and  
191 on the roof of the conceptacle (Figure 2E). The morphometric and fertility variables are summarised  
192 in Table 1.

193 *Table 1: Morphometric variables of the thalli, receptacles, aerocysts and gametes from the different*  
 194 *specimens.*

Location	Specimen length (cm)	Receptacle length (cm)	Aerocyst length (cm)	Oogonia length (µm)	Antheridia length (µm)	Number of released zygotes
Lido 1	27.49 ± 0.27	2.87 ± 0.42	1.03 ± 0.16	121.2 ± 30.0	30.5 ± 10.2	105.00 ± 2.02
Lido 2	26.60 ± 0.22	1.82 ± 0.48	0.77 ± 0.13	116.70 ± 26.8	26.6 ± 12.4	91.00 ± 2.61
Sassonia	20.40 ± 0.18	1.47 ± 0.30	0.64 ± 0.08	90.0 ± 20.0	20.0 ± 9.0	78.00 ± 3.02

195

# 196 *Recruits height and density in mesocosms*

197 Across maintenance in mesocosms, the average heights were  $2.10 \pm 1.13$ ,  $2.06 \pm 0.95$  and  $1.79 \pm 0.87$   
 198 mm, for recruits deriving respectively from Lido 1, Lido 2 and Sassonia. Recruits from Lido 1 and  
 199 Sassonia grew progressively over the 3 months (i.e., from month 1 to 2 and from month 2 to 3),  
 200 whereas those from Lido 2 grew from month 1 to month 2 (Figure 3A).

201 The average density was  $78.3 \pm 4.57$ ,  $67.5 \pm 3.66$  and  $47.5 \pm 3.51$  recruits per tile, respectively  
 202 for Lido 1, Lido 2 and Sassonia (Figure 3B). Recruits density decreased progressively over the 3  
 203 months (i.e., from month 1 to 2 and from month 2 to 3), for Lido 1 and Sassonia, whereas decreased  
 204 form month 2 to 3 for Lido 2 (Figure 3B).

205

# 206 *Recruits growth in the field*

207 When recruits were out planted in the field, they measured  $2.94 \pm 0.34$ ,  $2.49 \pm 0.27$  and  $2.49 \pm 0.25$   
 208 mm, respectively for Lido 1, Lido 2 and Sassonia. After 4 weeks in the field, the recruits continued  
 209 to grow reaching  $8.93 \pm 0.77$ ,  $7.10 \pm 1.26$  and  $4.38 \pm 0.40$  mm, respectively for Lido 1, Lido 2 and  
 210 Sassonia (Figure 4A). Two-way ANOVA revealed significant effects of “time” and “location” on  
 211 recruits heights ( $p < 0.05$ ; Table 2).

212

213

214 *Table 2: Results of ANOVA on recruits length and density.*

	<b>Factors</b>	<b>df</b>	<b>F</b>	<b>p</b>
<b>Recruit length</b>	Time	1	7.55	0.008
	Location	2	11.58	< 0.001
	Time x Location	2	1.14	0.33
<b>Recruit density</b>	Time	1	7.55	0.018
	Location	2	10.14	0.008
	Time x Location	2	1.35	0.29

215

216 When recruits were out planted in the field, the density were  $56.7 \pm 5.22$ ,  $53.20 \pm 5.20$  and  
 217  $27.1 \pm 4.43$  per tile, respectively for Lido 1, Lido 2 and Sassonia. After 4 weeks, the density decreased,  
 218 being  $26.3 \pm 3.79$ ,  $35.7 \pm 14.8$  and  $12.7 \pm 5.51$ , respectively in the structures with recruits from Lido  
 219 1, Lido 2 and Sassonia (Figure 4B). A significant effect of “time” and “location” was observed on  
 220 recruits density (ANOVA,  $p < 0.05$ ; Table 2).

221 The recruits showed a high survival % in the field, with  $59.0 \pm 1.2$  %,  $54.0 \pm 6.7$  % and  $39.0$   
 222  $\pm 2.7$  %, respectively for the structure Lido 1, for Lido 2 and Sassonia (Figure 4C). The Tukey test  
 223 revealed significant lower values for Sassonia than the Lido 1 and Lido 2 ( $p < 0.05$ ).

224

## 225 **Discussion**

226 Stranded individuals of *G. barbata* offer an opportunity to promote habitat restoration, producing  
 227 healthy and viable recruits. Indeed, stranded individuals can be successfully rescued and maintained  
 228 in the laboratory and, if their fronds bear fertile apices, they may produce vital embryos and zygotes.  
 229 In particular, we report here that after three months in mesocosms, the new recruits reached an average  
 230 height of 1-2 mm and an average density of 50-80 recruits on each tile used as substrate,  
 231 corresponding to ca. 13-21 new recruit per  $10 \text{ cm}^{-2}$ .

232 The use of stranded individuals represents a new approach to obtain vital recruits for  
 233 macroalgal restoration. Previous macroalgal restoration approached, indeed, were based on the  
 234 collection of the on *Cystoseria s.l.* *in situ* or their *ex situ* reproduction (or a combination of both). The  
 235 recruits growth rates (on average  $0.2\text{-}0.7 \text{ mm week}^{-1}$ ) and densities (up to 7 recruits  $10 \text{ cm}^{-2}$ ) reported

236 here from the stranded seaweed *G. barbata* are similar to those previously reported in the Adriatic  
237 Sea and in other Mediterranean regions using the standard approaches (Verdura et al. 2018; Savonitto  
238 et al. 2021; Orlando-Bonaca et al. 2022). The same applies when we compare the data presented here  
239 with previous studies conducted in the same area, i.e. the Conero Riviera (Central-eastern Adriatic  
240 Sea; Bianchelli et al. 2023a). Although we need further experiments to ascertain the efficacy of out  
241 planting interventions over the long term and at a large spatial scale, these findings suggest that this  
242 approach could represent a promising tool for restoration purposes.

243         The collected stranded fragments showed a large variability in the length of receptacles,  
244 aerocysts and gametes, but also in the number of released zygotes. During the maintenance in  
245 mesocosms and in the field after the outplant, we detected a difference in the recruits growth and  
246 density depending on the sampling location. Such differences could be due to the variability among  
247 different populations or some specimens may have been exposed to desiccation for a longer time,  
248 limiting their reproductive capacity. To better understand the driver of these difference, further studies  
249 are needed to ascertain which are the factors influencing the reproductive potential of stranded  
250 individuals, which may rely on source population health and distance, maturity and fertility of the  
251 individuals, floating and stranding period, but also environmental conditions, as season or water  
252 temperature (being the gametes' release temperature-dependent).

253         Moreover, we don't know the exact location of the source population of individuals stranded.  
254 We can hypothesize that the individuals coming from populations facing the shore, likely colonizing  
255 the breakwaters nearby. This aspect is particularly important, since *Cystoseira s.l.* populations are  
256 often fragmented and genetically disconnected also at small spatial scales, and connected by very low  
257 migration rates, along Mediterranean coasts (Buonuomo et al. 2016; Verdura et al. 2023). In turn, this  
258 has important implications for the identification of relevant conservation and management measures:  
259 each population, indeed, should be considered as separated units with dedicated conservation and  
260 restoration efforts (Riquet et al. 2021). On the other hand, dispersal of these species could be  
261 facilitated by rafting (Riquet et al. 2021). Moreover, detachment, rafting and stranding of adults may

262 be the effect of extreme events, such as heatwaves or storms, which are predicted to be more and  
263 more frequent in the future, due to climate change (Blanfuné et al. 2019).

264 The observed variability among *G. barbata* stands is consistent with previous studies, which  
265 reported two main morphologies: i) long, sickle-shaped receptacles with numerous chained aerocysts,  
266 and ii) small, oval or spindle-shaped receptacles with no or few aerocysts (Orlando-Bonaca et al.  
267 2022). Different morphologies and reproductive success suggest the presence of a morphological  
268 plasticity of *G. barbata*, possibly driven by different ecological conditions (Orlando-Bonaca et al.  
269 2022). Testing the reproductive potential and recruits performance from different *G. barbata*  
270 populations could be suggested as a complementary action (Cebrian et al. 2021) to enhance the  
271 success of *G. barbata* restoration.

272 This study shows the feasibility of using fertile thalli stranded on the beach to obtain recruits  
273 for restoration, without damaging natural populations and habitats (Ferretto et al. 2021). In this  
274 regard, also stranded stems of *Posidonia oceanica* are used for its restoration (Piazzi et al. 2021). This  
275 approach has the double advantage of obtained recruits without any damage to the natural  
276 populations, and promoting a sort of “circular economy” of the re-use of the stranded  
277 seagrass/macroalgae that would be otherwise lost. This approach showed a high recruitment success  
278 and growth during the cultivation in mesocosms and after the outplant. Indeed, the recruits continued  
279 to grow in length and showed a survival up to 80% after one month in the field.

280

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291

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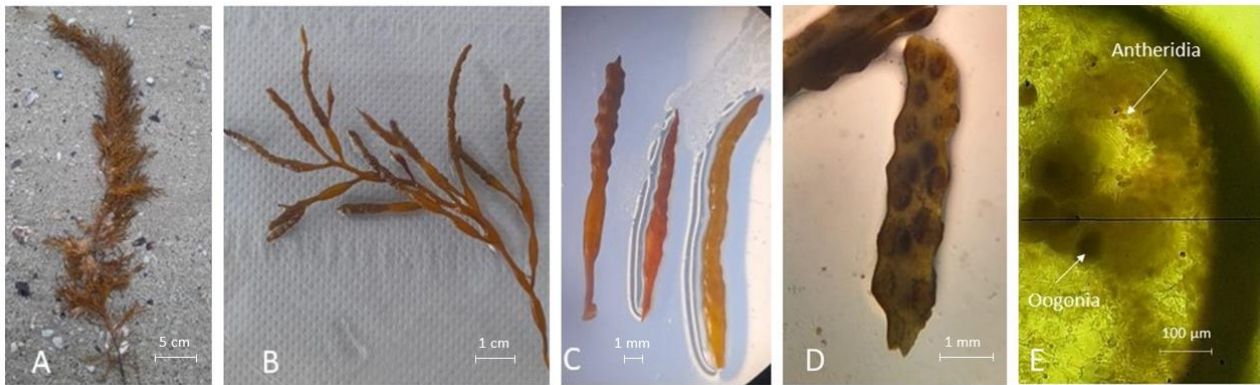
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393 *Figure 1: A) Study area and location of the sampling locations in the Adriatic Sea; B) locations where the*  
394 *stranded fragments of* *Gongolaria barbata* *were collected. Sassonia*  $43.84^{\circ}$  *N –*  $13.02^{\circ}$  *E; Lido 1 and Lido 2*  
395  $43.85^{\circ}$  *N -*  $13.01^{\circ}$  *E. The distance between Sassonia beach and Lido 1 is about 750 m, whereas Lido 1 and Lido*  
396 *2 are about 250 m apart.*

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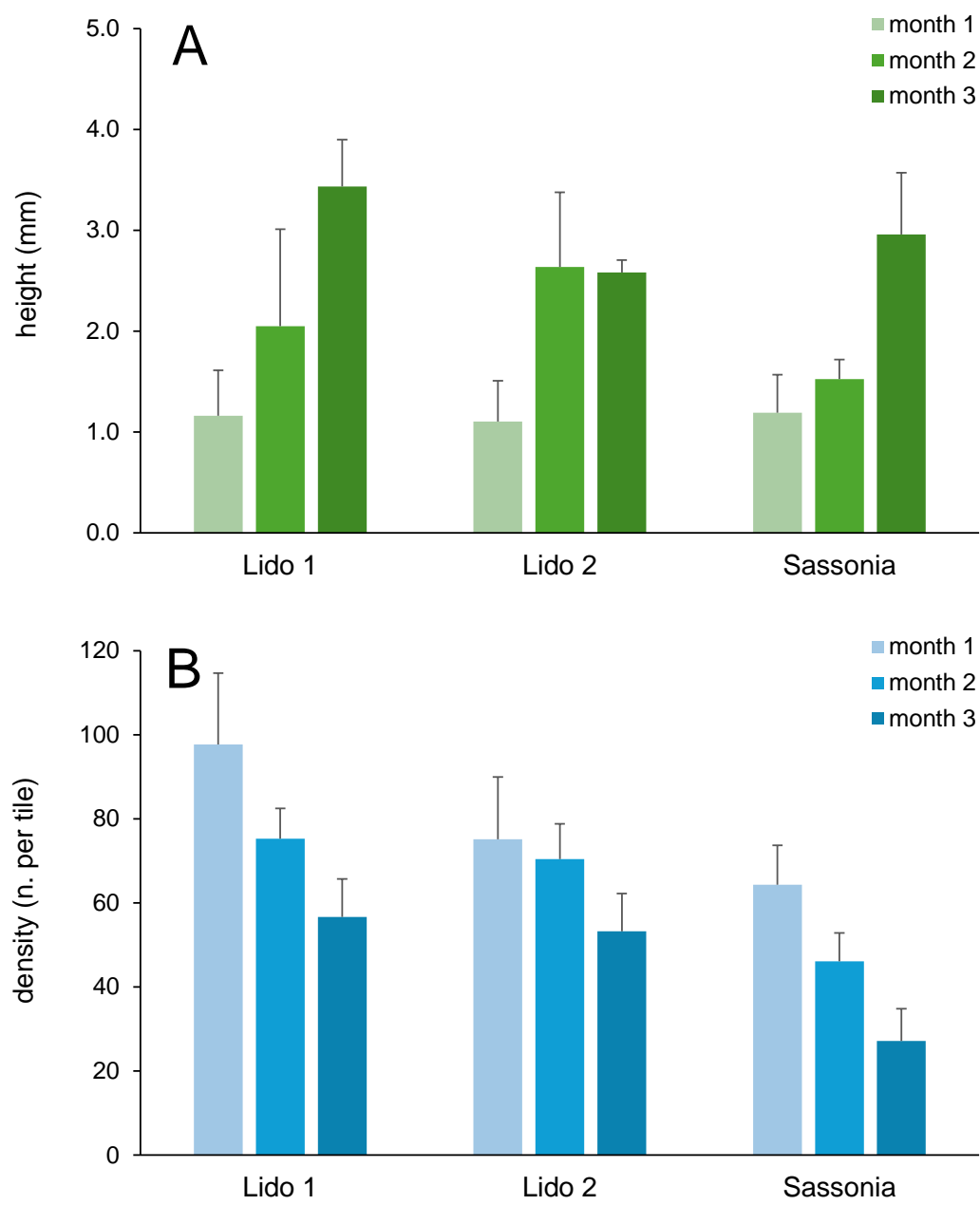


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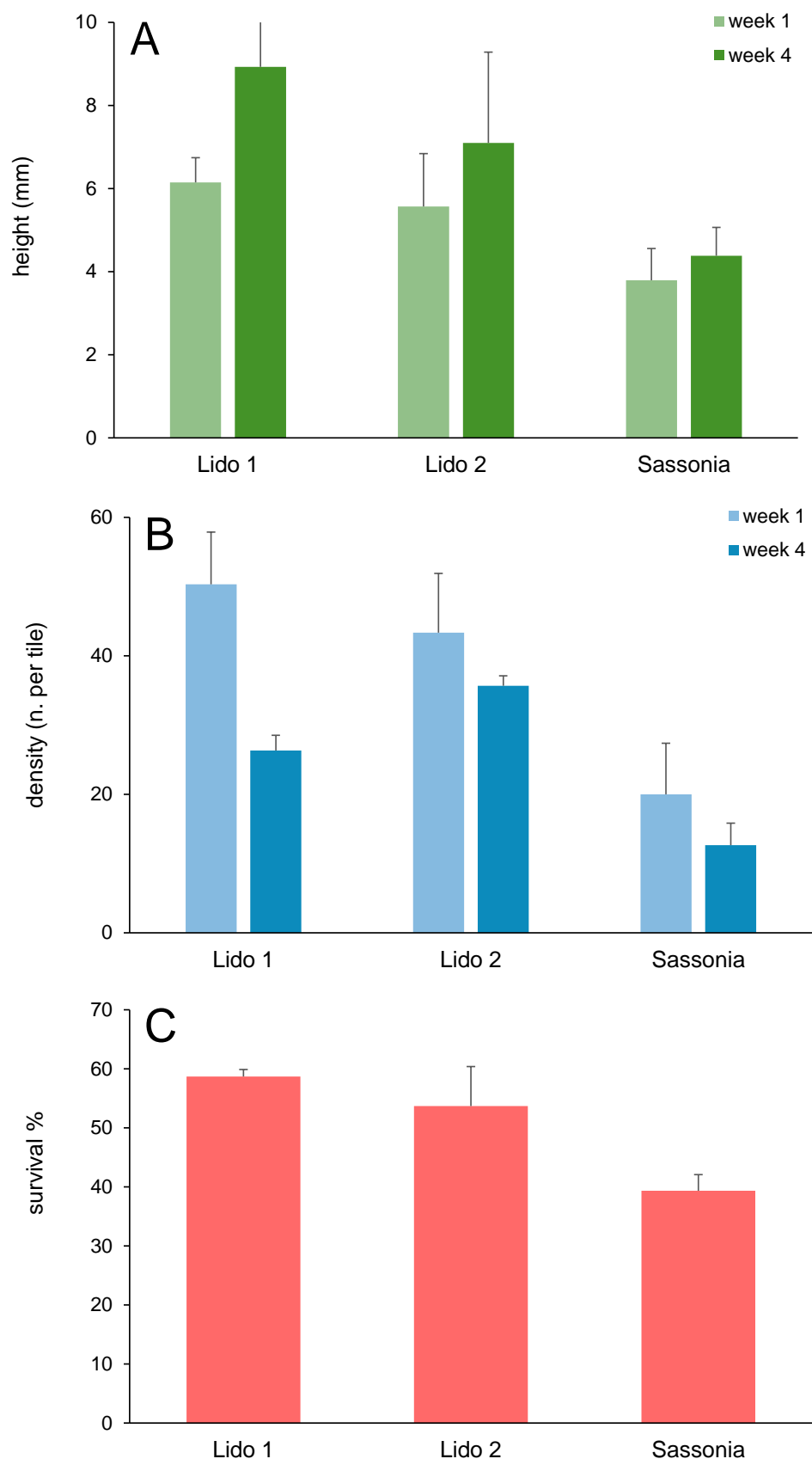
400 *Figure 2: A) Stranded specimen of G. barbata; B) Receptacles on terminal branchlets; C) Variability of*  
401 *receptacles; D) Longitudinal section of a receptacle showing the conceptacles arranged in parallel rows; E)*  
402 *Cross section of a receptacle showing oogonia and antheridia located in the conceptacle.*

403

404



407 *Figure 3: Mean heights (A) and density (B) of Gongolaria barbata recruits on tiles placed below the*  
408 *fertile adults, across the 3 months in mesocosms. Data are reported as mean  $\pm$  standard error.*



417 *Figure 4: Mean heights (A), density (B) and survival % (C) of Gongolaria barbata recruits after*  
418 *outplant at sea, across the 4 weeks of monitoring. Data are reported as mean  $\pm$  standard error.*