



## New hatchery methods for efficient spore use and seedling production of *Palmaria palmata* (dulse)

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1 New hatchery methods for efficient spore use and seedling production of *Palmaria palmata* (dulse)

2

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6 Key words: Dispersal, fertilization, hatchery, tetraspores, reattachment, spore seeding

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8

9 Abstract

10 *Palmaria palmata* (dulse) is a high valued rhodophyte; nevertheless, its hatchery methods are  
11 underdeveloped. New hatchery methods are required to improve the spore use efficiency and seeding  
12 quality, which are important benchmarks for a viable cultivation. This study investigated a method using  
13 vertical seeding tanks (exp. 1), hemispherical agitation and flow-through conditions to improve spore  
14 dispersal on net substrates. Tanks were inoculated with different amounts of sori tissue, which sporulated in  
15 three consecutive seeding periods. The results demonstrated significant effect of seeding period where 5-15  
16 g FW sori could be used to seed three nets (~126 m rope) over the course of nine days providing a density  
17 up to 10 seedlings cm<sup>-1</sup> after 32 days. The effluent spores were collected in detaining tanks and germinated  
18 into a propagule mix of female and male gametophytes. The propagule mix was efficient as a secondary  
19 seeding inoculum, as propagules were able to reattach to substrates up to 39 days after their release as  
20 spores (exp.2). Adding male gametes to the propagule mix and spore seeded ropes was tested as a relevant  
21 hatchery step to activate female gametophytes and significantly resulted in more than a doubling of  
22 seedlings (exp.3). This study present new methods and strategies to improve spore use efficiency and to  
23 obtain an equal spore dispersal on net substrates for hatchery production of *P. palmata*.

24

## 25 1. Introduction

26 The rhodophyte *Palmaria palmata* (L.) F. Weber and D. Mohr has traditionally been used for human  
27 consumption with records dating back to the 9<sup>th</sup> century (Mouritsen et al. 2013). The emerging evidence of its  
28 umami flavor (Mouritsen et al. 2013), bioactive and health properties of protein hydrolysates (Harnedy et al.  
29 2014; Admassu et al. 2018), water extracts (Lee et al. 2017) and biorefined compounds (Schiener et al.  
30 2017) has renewed interest in the use of *P. palmata*, resulting in increased focus on its cultivation. *P.*  
31 *palmata* can be cultivated in tanks using vegetative growth (Morgan and Simpson 1981; Pang and Lüning  
32 2004; Matos et al. 2006; Corey et al. 2014), but the prospects of cultivating the species from tetraspores has  
33 lately received increasing attention in Europe (Edwards and Dring 2011; Werner and Dring 2011; Sanderson  
34 et al. 2012). However, despite studies founding the essential understanding of the life cycle (van der Meer  
35 and Chen 1979; van der Meer and Todd 1980) and cultivating *P. palmata* from spores (Browne 2001;  
36 Sanderson 2006; Werner and Dring 2011; Grandorf Bak 2019; Schmedes et al. 2019), the methodology for  
37 large-scale hatchery production in Europe is still in its infancy.

38 Essentially, the use of spores for cultivation of *P. palmata* is based on three steps; 1) the collection or  
39 induction of sori, 2) the release and dispersal of spores and 3) the spore attachment and growth; and it is the  
40 efficiency and success of these three steps that determines the usefulness of a given hatchery protocol. As  
41 the steps are interlinked, the initial handling of sori, which represent only 8-10% of the total frond area during  
42 the peak season of fertility (Werner and Dring 2011), is crucially important. Currently, the most commonly  
43 used hatchery protocol to produce *P. palmata* seeded substrates is a flat tank using a 1:1 areal coverage of  
44 substrate with sori and a three days spore release duration. However, this method requires a large amount  
45 of sori, and furthermore, results in poor dispersal and high mortality (60-90%) of the spores (Werner and  
46 Dring 2011). Also, by the using this method, it is often observed that a considerable amount of the spores  
47 settle on tank surfaces instead of the intended cultivation substrate (personal observation).

48 Taking into account these results and observations, it is clear that there is a bottleneck in spore use  
49 efficiency within *P. palmata* hatcheries. This particular matter was addressed in a recent study suggesting a  
50 GMA-seeding method (Germination, Maceration, and Agitation method) as an alternative seeding method for  
51 *P. palmata* (Schmedes et al. 2019). This study found that germinated propagules (i.e. a mix of spores and  
52 seedlings) of *P. palmata* were able to establish discoid reattachment to a substrate after forced de-  
53 attachment and maceration, resulting in high settlement efficiency as well as a high dispersal of the  
54 propagules. However, the extensive use of this seeding method is still unknown. Besides the use of this  
55 seeding strategy, the use of male gametes of *P. palmata* to fertilize female gametophytes has been  
56 suggested as a potential hatchery step to optimize the hatchery production (Mine and Tatewaki 1994; Le  
57 Gall et al. 2004; Schmedes et al. 2019). When using sori for spore-seeding, a mixture of male and female  
58 spores develop after attachment, however, only the males will develop into a harvestable thallus unless a  
59 fertilization step, enabling zygote formation in the female gametophyte, is included. Theoretically, this would  
60 double the seedling density, as the crustose-like female gametophyte will develop into a sporophytic thallus  
61 after the zygote. Yet, this is still uninvestigated under relevant hatchery conditions.

62 Because of the continued challenges in producing *P. palmata* sporophytes and gametophytes, this present  
63 study investigated hatchery methods and strategies based on Schmedes et al. (2019) to further improve the

64 utilization of spores for cultivation of *P. palmata* on substrates. Five strategies to improve the hatchery  
65 protocol were tested: 1) A vertical seeding tank in flow-through condition agitated with air bubbles to promote  
66 dispersal of spores. 2) The effect of different amount of sori during the seeding phase. 3) The effect of using  
67 the same sori material in three consecutive seeding periods on the spore germination and seedling density.  
68 4) The use of detained spores as a propagule seeding inoculum, based on the GMA-method. 5) The effect of  
69 fertilization on the number of attached seedlings under relevant hatchery conditions.

70

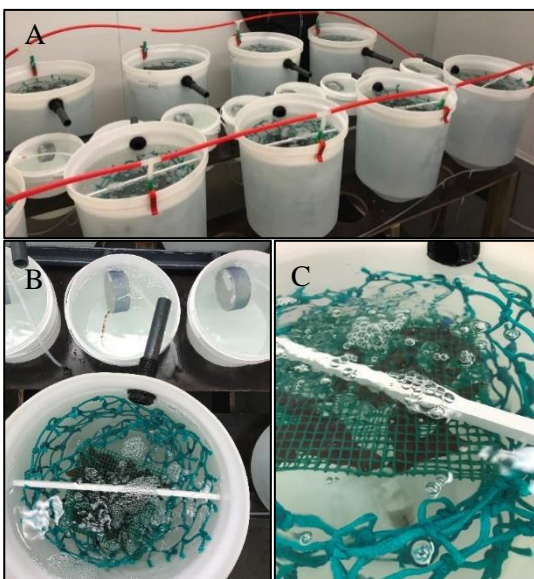
## 71 2. Materials and methods

72 Three experiments were set up to test the five improvements in hatchery techniques described above.

73 Experiment 1 investigated a new flow-through seeding system for improved seeding dispersal, additionally  
74 using different sori amounts in consecutive seeding periods as a means to improve sori use. This experiment  
75 addressed the first three steps of hatchery improvements. Experiment 2 covered step four, optimizing the  
76 use of detained spores, and finally, experiment 3 exploited the opportunity to include fertilization as a means  
77 to improve spore use, addressing step five of hatchery improvement techniques.

### 78 2.1. Experiment 1: spore seeding and sori use.

79 Nine conical tanks (polyethylene, 30 L) were set up as seeding tanks in a parallel flow-through system with a  
80 water flow of 0.5 L min<sup>-1</sup> (fig. 1A) in a 10 °C cold room. Air bubbles from the bottom (1.2 L min<sup>-1</sup>) bubbled  
81 through the sori package near the surface (fig. 1C), generated a hemispherical circulation of the water  
82 allowing the spores to disperse and aerated the tank volume. Water for the entire system was circulated from  
83 a reservoir tank with a circulation pump and exchanged (10% of total volume) on a daily basis. No nutrients  
84 were added. From each seeding tank, an outlet, placed diagonal to the inlet, led the effluent water directly  
85 into a 2 L spore-detaining tank (SDT) (fig. 1B). Here, effluent spores aggregated on the bottom, while water  
86 was led out in the top and back to the reservoir tank through a nylon filter (15 µm) for UV treatment (60 W).  
87 Spores detained in the SDTs were used in experiment 2.

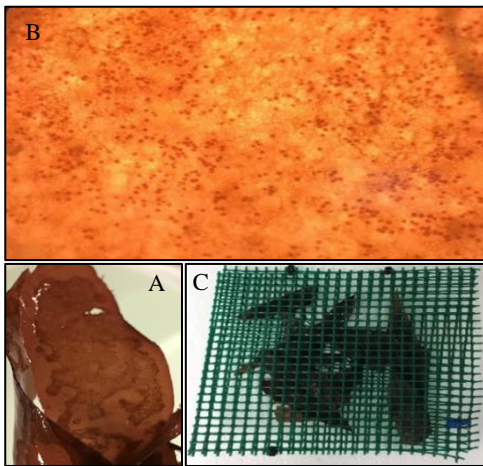


88

Figure 1.

89 Twenty-seven pieces of net (polypropylene, 0.25\*1.40 m,  $\phi$ =5-7mm) were prepared as cultivation substrate  
90 by soaking them in lukewarm tap water a month before, then kept in clean seawater for seven days before  
91 use. At the experimental start, the nets were submerged in the seeding tanks as a two-layered net spiral (fig.  
92 1B).

93 Fertile *P. palmata* tetrasporophytes and male gametophytes were collected 4 January 2019 in the intertidal  
94 zone near Fornæs light house, Denmark (56.443534N, 10.958985E) and kept in running seawater (4 °C) in  
95 dim natural light ( $\sim 15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , PAR). On 10 January, 218 fertile fronds were rinsed in 0.2  $\mu\text{m}$   
96 filtered and sterilized seawater (fig. 2A) before being desiccated for 20 hours at 5 °C in darkness. Presence  
97 of ripe sporangia was verified by inspection (fig. 2B). Triplicates of three groups of sori amount (5, 10 or 15 g  
98 FW; fresh weight) were prepared as sori packages by placing the tissue between two layers of plastic net  
99 (15\*15 cm, fig. 2C).



100 Figure 2.

101 The experiment started 11<sup>th</sup> of January 2019 by adding a net and a sori package (3 replicates of 3 sori  
102 amounts) to each of the nine tanks. The seeding ran over a course of nine days, keeping the same sori  
103 package in the tanks for all nine days but exchanging the nets with new ones every third day to test the  
104 potential of consecutive seeding periods (Day 0-3, Day 3-6, Day 6-9) using the same sori. During seeding,  
105 the surface irradiance was 15-22  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 10:14 h L: D. At the end of each consecutive seeding  
106 period, the nine nets were labelled and transferred to nursery tanks containing 300 L enriched seawater (0.2  
107  $\mu\text{m}$  filtered); 10% strength of Varicon Aqua Cell-Hi F2P, a F/2 nutrient media with vitamins based on Guillard  
108 and Ryther (1962). Air stones provided water agitation in the tanks after three days. Irradiance was 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (10:14 h L: D), but raised to 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  from week 2.

110

#### 111 *Data acquisition*

112 The net-seeding efficiency using sori in the flow-through system was calculated by equation 1:

113 
$$\text{Net seeding efficiency} = \frac{\# \text{ spores on net}}{\# \text{ Total spores}(\text{net} + \text{detained})} * 100\%$$

114 This was carried out by counting the number of attached spores and seedlings on a 4 cm net subsample for  
 115 each of the 9 nets (n=3) three days after each seeding period. Additionally, the nets were counted at day 19  
 116 and 32 after each of the consecutive seeding periods to compare spore and seedling density within and  
 117 between groups over time. The subsamples were taken from the same position regarding the in, - and outlet  
 118 of the tanks on each net spiral. To verify an even spore dispersal on the entire net, additional subsamples  
 119 were taken from the 10 g sori batch in the first seeding period (Day 0-3). From each net (n=3), three  
 120 subsamples were taken from the bottom and top part of the net (N=9) and tested for unequal variance using  
 121 Levene's test. Similarly, by counting spores on subsamples (N=21) from all around one net spiral in the  
 122 same vertical level the spore dispersal was assessed. By verification, we extrapolated the spore and  
 123 seedling densities for each counting day and calculated the germination success by equation 2:

$$124 \quad \text{Germination (\%)} = \frac{\text{\# Seedlings}}{\text{\#Total propagules (spores + seedlings)}} * 100\%$$

125 The total number of detained spores in each SDT after a seeding event (fig. 3A) was estimated based on  
 126 subsample counting, by the following process: 1) A homogenous spore mix (fig. 3B) was prepared by  
 127 reducing the SDT volume, dislodging the spore aggregates and macerating the solution for 30 seconds with  
 128 a kitchen blender. 2) A 10 mL subsample was transferred to a gridded petri dishes (fig. 3C) and added 20  
 129 mL enriched (10% F/2) seawater. 3) The spores were kept at 5 °C and  $5 \pm 2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (10:14 h  
 130 L:D) for three days before five random fields (each  $0.0035 \text{ mm}^2$ ) were photographed and counted. The total  
 131 number of spores was estimated by factor multiplying the mean count of subsamples ( $\text{STD}_{\text{VOLUME}} * 10 \text{ mL}^{-1}$   
 132 subsample and petri dish area \*  $0.0035 \text{ mm}^2$ ).

133

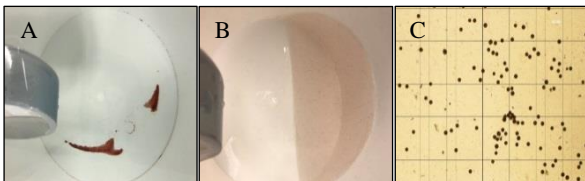


Figure 3.

134

135

## 136 2.2. Experiment 2: Mixed propagules as seeding inoculum

137 In Experiment 2, the propagules germinated from detained spores from experiment 1 were tested as seeding  
 138 inoculum, according to the GMA-method (Schmedes et al. 2019). After subsampling for petri dish cultures in  
 139 experiment 1, the rest of the macerated spore solution from the last seeding period (Day 6-9) was poured  
 140 into a plexiglas containers (5 L), settled on the bottom (fig. 4A) and cultivated at  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$   
 141 (adjusted to  $5 \pm 2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , after ten days) and 5 °C. After one day, enriched seawater and  
 142 germanium-oxide (10% F/2 + 1 mg L<sup>-1</sup> GeO) was added and after six days, an air stone was put for aeration.  
 143 The detained spores germinated into a mixture of propagules (i.e. spores and gametophytes) at these  
 144 conditions before use.

145 Twenty-seven days after the initiation of Day 6-9 seeding period, some of the propagules on the bottom were  
 146 dislodged (Fig. 4B), suspended in 400 mL enriched (10% F/2) seawater and macerated to break propagule



147 aggregates (fig. 4C) according to Schmedes et al. (2019). The propagules were photographed before and  
148 after the maceration treatment (fig. 4D, E). A total of 20 mL of the propagule mix was added to each of 10  
149 beakers (n=10) containing 400 mL enriched (10% F/2) seawater and a piece of rope (10 cm) standing  
150 upright in the beaker to test the ability of the propagules to reattach. The rope was made by untangling  
151 several net meshes and cut and cut into pieces. Immediately, 1 mL subsamples were withdrawn and the  
152 concentration of macerated propagules was estimated to  $259 \pm 8$  spores  $\text{mL}^{-1}$  and  $422 \pm 13$  seedlings  $\text{mL}^{-1}$   
153 (mean  $\pm$  SE, n=4). The beakers were constantly agitated from the bottom via air bubbles ( $2.5 \text{ L min}^{-1}$ ) to  
154 promote the dispersal of the propagules.  
155

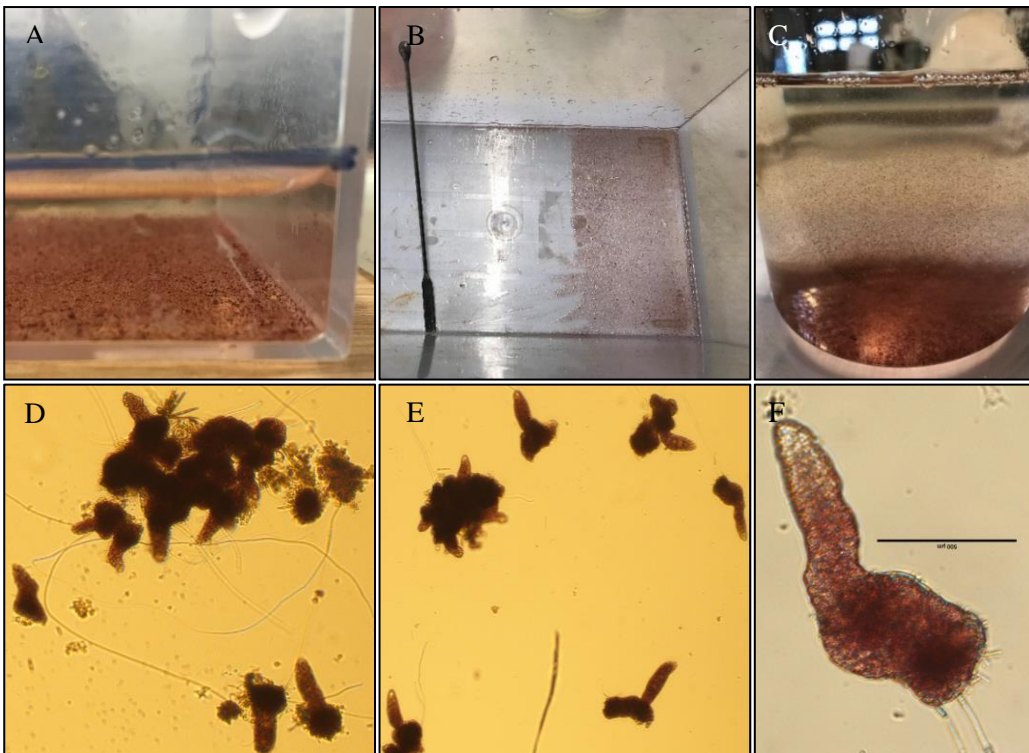


Figure 4.

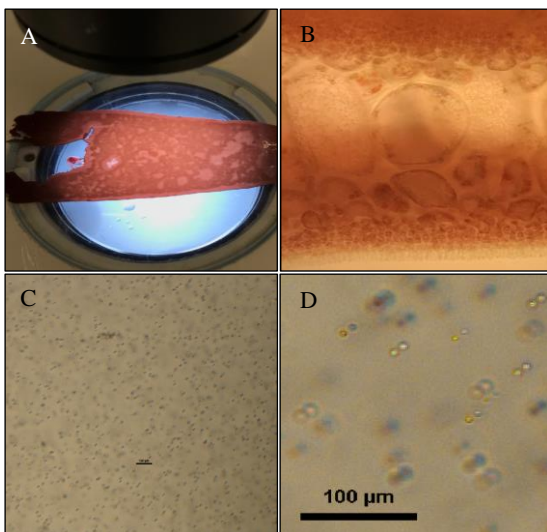
156  
157  
158 After 3 days inoculation (“agitation period day0-2”), the maximum density of attached spores and seedlings  
159 was estimated by counting the three densest subparts (0.5 cm) for each rope, after which the ropes were  
160 transferred to 20 L tanks at the similar conditions until they were counted again at day 10. After transferring  
161 the ropes pieces out of the beakers, the propagule mix that maintained in the beakers under same conditions  
162 until day 10, where concentration (mean of technical replicate  $\pm$  SE) of spores ( $237 \pm 7$ ) and seedlings  
163 ( $394 \pm 9$ ) seedlings was similar to the starting concentration (two-sampled t-tests:  $t(4)$ ,  $p=0.086$ ,  $p=0.131$ ,  
164 respectively). Then, new pieces of rope were added for 3 days inoculation to see how long after the  
165 dislodgement and maceration the propagules would be able to reattach (“agitation period day10-12”). Also,  
166 at day 10, new beakers (n=10) were set up under same conditions using freshly dislodged and macerated  
167 propagules to see if the age of the propagules (27 vs. 37 days) at dislodgement affected the ability to  
168 reattach. The concentration of propagules ( $250 \pm 20$  spores and  $396 \pm 22$  seedlings) was similar (two-sampled  
169 t-tests:  $t(4)$ :  $p = 0.584$ ,  $p = 0.341$ ) with the other start concentrations.

170 2.3. Experiment 3: Including fertilization in hatchery

171 The effect of fertilizing female gametophytes was tested by adding male gametes to propagules cultured in  
172 petri dishes and spore-seeded ropes cultured in beakers. A solution of propagules ( $349 \pm 26$  propagules  $\text{mL}^{-1}$ ,  
173  $n = 9$ , technical replicates) was prepared from the same detained spore batch as used in experiment 2 by  
174 dislodging and macerating after 11 days of germination. A total of 15 mL propagule solution was then poured  
175 into each of 18 petri dishes and filled with additional 10 mL enriched (10% F/2) seawater and kept in  $15 \pm 3$   
176  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  PAR (12:12 h L:D) irradiance at 5 °C.

177 At day 1, a solution of male gametes was prepared and 15 mL of this solution was added to nine petri  
178 dishes, containing the propagule solution. Nine other dishes received 15 mL seawater (10% F/2) as a  
179 control. The male gamete solution was prepared by desiccating (3 h, dark at 5 °C) thirty fertile male  
180 gametophytic fronds (22.4 g FW, see fig. 5A and 5B) before being rehydrated (1 h,  $5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$   
181 PAR) in an agitated volume of 400 mL seawater + 0.4 mL GeO (1g/L). The male gametes ( $\text{Ø} \sim 5 \mu\text{m}$ ) were  
182 visible using high magnification (fig. 5C and 5D). The concentration of gametes was estimated to be  $\sim 1000$   
183 gametes  $\text{mL}^{-1}$ , using a Neubauer cell counting chamber. After adding, the cultures were mixed on a stirring  
184 table for 30 min at 160 rpm. At day 4 and 6, the petri dish volumes were exchanged with new additions of  
185 male gamete solution (5 mL,  $\sim 10^6$  gametes  $\text{mL}^{-1}$ ).

186 For each petri dish, spores and seedlings were counted in five random fields (each  $0.0035 \text{ mm}^2$ ) at day 0, 3,  
187 10 and 19 and the number of seedlings were adjusted to the spore count of previous counting day.



188 Figure 5.

189 In addition, we assessed the effect of fertilization on the seeded rope pieces (Exp. 2). This was carried out in  
190 beakers (1.8 L) kept at 5 °C, in which 4 cm pieces of seeded rope were tumbled by air bubble agitation  
191 ( $n=11$ ). The pieces were excised from “Day 3-6 and 10 g sori” nets (Exp. 1) and nursed for 16 days at 10 °C,  
192 before being transferred to the beakers. This was followed by adding 40 mL solution containing male  
193 gametes to the beakers, while a control group was added the same enriched seawater (10% F/2) with no  
194 gametes ( $n=11$ ). The numbers of spores and seedlings was counted at day 1, 3, 6, and 12 and the seedling  
195 number was presented as relative to spore number.



## 196 2.4. Statistics

197 For all data sets, Shapiro Wilks test was used to check normality and Levene test was used to check  
198 variance homogeneity. The analysis was carried out using SAS, JMP 13, using a significance level of 0.05.  
199 When sufficient, data were log-transformed to ensure homogeneity of variance and normal distributed  
200 residuals and ANOVAs were conducted to compare the main effects. In case of none normal distribution or  
201 homogeneity of variance, the Wilcoxon two-sample test was used. All data are given as mean  $\pm$  standard  
202 error (SE), unless stated otherwise.

203 Experiment 1. In a factorial design, nets were manipulated to be in one of nine groups forming the  
204 combination of consecutive seeding periods (3 levels; Day 0-3, Day 3-6, Day 6-9) and sori amount (3 levels;  
205 5, 10, 15 g FW), and 2-way ANOVA (2w-an), including the interaction term, using log-transformed data was  
206 conducted to compare effects on spore and seedling numbers attached to the nets, the number of detained  
207 spores in STDs, the spore-seeding efficiency as well as the germination success (%) between all groups,  
208 followed by Tukey's HSD post hoc test. The Wilcoxon two-sample test was used to assess for any significant  
209 change in mean densities of spores and seedlings across the counting days within each factorial group.

210

211 Experiment 2. Datasets were log-transformed to ensure normal distribution, but spore counts did not obey  
212 homogeneity of variance (Levene,  $p < 0.0208$ ). Hence, the Wilcoxon test was used to assess for significant  
213 difference in mean settlement density at each count day.

214 Experiment 3. For the petri dish cultures, the effect of adding male gametes on the number of seedlings  
215 adjusted to the previous spore count was compared using a two-tailed Student's t test ( $n=11$ ). For the rope  
216 cultures, the univariate repeated measures ANOVA was used to test effect of the between factor (two levels;  
217 male gametes vs. control) and the within factors (time; four levels) on the adjusted number of seedlings.  
218 Datasets were normal distributed and displayed equal variance after log-transformation. The Mauchly's  
219 sphericity test:  $\chi^2(5) = 8.3513$ ,  $p = 0.1379$  allowed to report the p-value of the F test.

220

## 221 3. Results

### 222 3.1. Experiment 1: spore seeding and sori use

223 Spores settled on all nets during the consecutive seeding periods and for all amount of sori used (fig. 6A-C).  
224 Spores were present on all counting days, while seedlings (fig. 6D-F) were only present from counting day  
225 19 and onwards. On counting day 3, the number of spores attached to the nets was significantly affected by  
226 seeding periods (2w-an:  $F_{2,8} = 7.2461$ ,  $p = 0.0049$ ), the second (fig. 6B) and third (fig. 6C) seeding period  
227 (day 3-6 and day 6-9) showing similar and significant higher spore densities (Tukey's:  $p = 0.0036$ ) than the  
228 first seeding period (day 0-3) (fig. 6A). Also, the sori amount used for seeding, significantly affected the  
229 number of attached spores (2w-an:  $F_{2,8} = 4.0989$ ,  $p = 0.0341$ ), showing significant difference between high  
230 and low sori amounts (Tukey's,  $p = 0.0109$ ). On counting day 19, the number of spores (fig. 6A-C) and  
231 seedlings (fig. 6D-F) as well as the spore germination success (fig. 6G-I) was similar for all groups (2w-an:  
232  $p > 0.5606$ ). On counting day 32, the spore settlement was still similar across all groups (2w-an:  $p > 0.1179$ )

233 (fig. 6A-C), whereas the number of seedlings (fig. 6D-F) was significant affected by seeding period (2w-an:  
 234  $F_{2,8} = 6.1718$ ,  $p = 0.0091$ ), showing similar seedling density in second and third period (Tukey's,  $p = 0.8110$ ),  
 235 both significantly higher than the first seeding period (Tukey's,  $p = 0.0105$ ,  $p = 0.0383$ , respectively). At day  
 236 32, the average seedling density was  $36 \pm 8$  seedlings per 4 cm. However, the germination success (fig. 6G-I)  
 237 up to 50-80% turned out to be similar between seeding periods (2w-an:  $F_{2,8} = 3.0829$ ,  $p = 0.0706$ ) and sori  
 238 groups (2w-an:  $F_{2,8} = 0.1873$ ,  $p = 0.83$ ). Within each seeding period (fig. 6A-C), the spore count decreased  
 239 significantly over time (Chi-squared:  $\chi^2(9) = 10.14$ ,  $p = 0.0063$ ), whereas the increase in seedling density  
 240 was only significantly on nets produced in second (Mann-Whitney two-sample test:  $p=0.0030$ ) and third ( $p =$   
 241  $0.0295$ ) seeding periods.

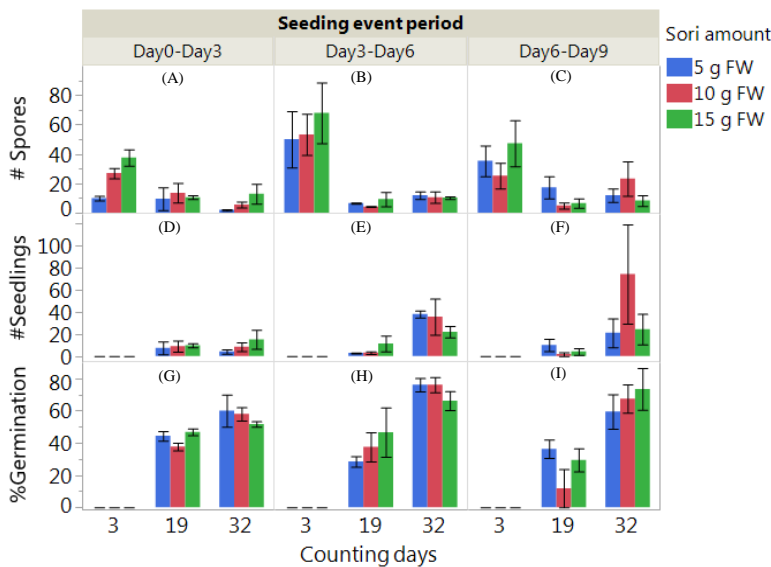


Figure 6.

242

243 The additional rope pieces sampled to assess whether settlement was homogeneous dispersed across the  
 244 entire net, showed that this was the case. Both spores and seedlings showed equal variance between top  
 245 and bottom of the nets (spores:  $p = 0.17$ , seedlings:  $p = 0.40$ ) and means (Mann-Whitney two-sample test:  $p$   
 246  $= 0.40$ ). Furthermore, the densities showed equal variance horizontally around the net spiral (spores:  $p =$   
 247  $0.23$ , seedlings:  $p = 0.33$ ). Hence, the number of spores settled on rope pieces cut from the nets was  
 248 extrapolated to whole nets (fig. 7A-C) and summed with the number of detained spores (fig. 7D-F) to  
 249 calculate the total number of released spores. This number was then used to estimate a net seeding  
 250 efficiency of the system (fig. 7G-I) by equation 1. The net seeding efficiency of the agitated, flow-through  
 251 seeding tank system using sori packages was in average  $16 \pm 1.6\%$  (fig.7G-I) and not affected by either  
 252 seeding period or sori amount (2w-an:  $p > 0.28$ ). This indicates that app. 84 % of the released tetraspores  
 253 were washed out and detained (fig. 7D-F).

254 Effluent spores aggregated as red plumages on the bottom of all the SDTs, and were visible within 3 hours  
 255 during the first seeding period (Day 0-3). The number of detained spores from the flow-through system (fig.  
 256 7D-F) was significantly affected by seeding period (2w-an:  $F_{2,8} = 5.66$ ,  $p = 0.0124$ ), where second period  
 257 provided higher detained spore yield compared to the third period (Tukey's:  $p = 0.0117$ ) and similar spore  
 258 yield as first period ( $p = 0.6593$ ). The amount of sori used also affected the number of detained spores

259 significantly (2w-an:  $F_{2,8} = 5.07$ ,  $p = 0.0178$ ) where 15 g sori provided higher yield compared to 5 g sori  
 260 (Tukey's:  $p = 0.0175$ ). In total, the amount of released spores (detained + attached to nets) from the three  
 261 different sori packages during nine days of sporulation was significantly higher for the 15 g sori group  
 262 (1,300,563±71,639), compared to the 10 g sori (959,697±69,618) and the 5 g sori group (522,230±61,869)  
 263 spores.  
 264

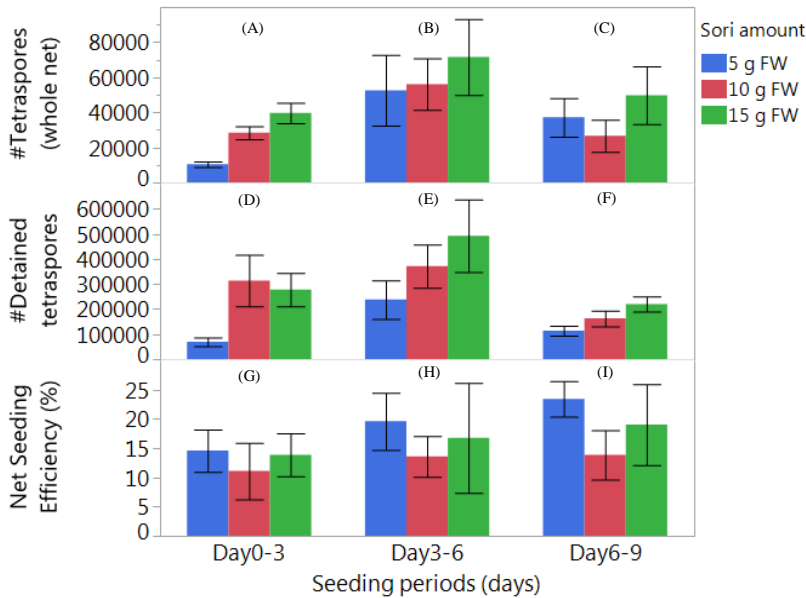


Figure 7.

265  
 266  
 267

### 3.2. Experiment 2: Mixed propagules as seeding inoculum

268 On counting day 3, the number of reattached spores (fig.8A vs. 8B) and seedlings (fig. 8C vs. 8D) was  
 269 significantly higher, when the inoculum was dislodged at day 27-29 compared to day 37-39 (Mann-Whitney  
 270 two-sample test (10),  $p < 0.0001$ ). Furthermore, the number of reattached spores and seedlings on ropes  
 271 exposed to different agitation periods (Day 0-2 vs. Day 10-12) was significantly different ( $p < 0.0001$ ,  $p =$   
 272  $0.0012$ , respectively). After nursing these ropes for additional seven days (counting day 10), the effect of  
 273 agitation period on the density of spores and seedlings remained significant ( $p = 0.0002$ ,  $p = 0.0009$ ). In  
 274 contrast, the effect of inoculum age at dislodgement was insignificant on the spore density ( $p = 0.0697$ ) and  
 275 seedling density ( $p = 0.9938$ ).

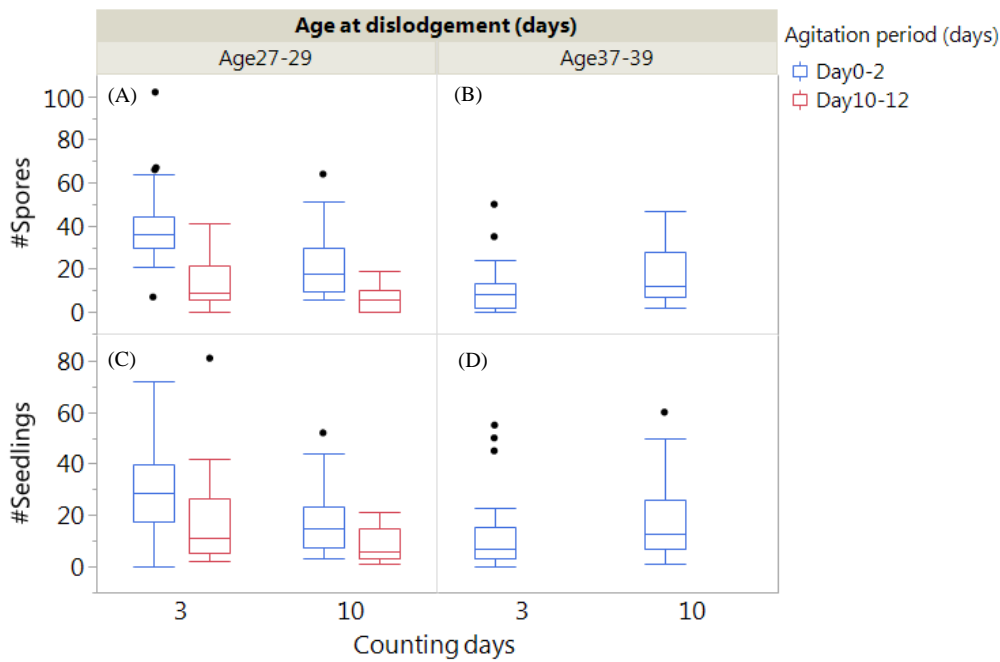


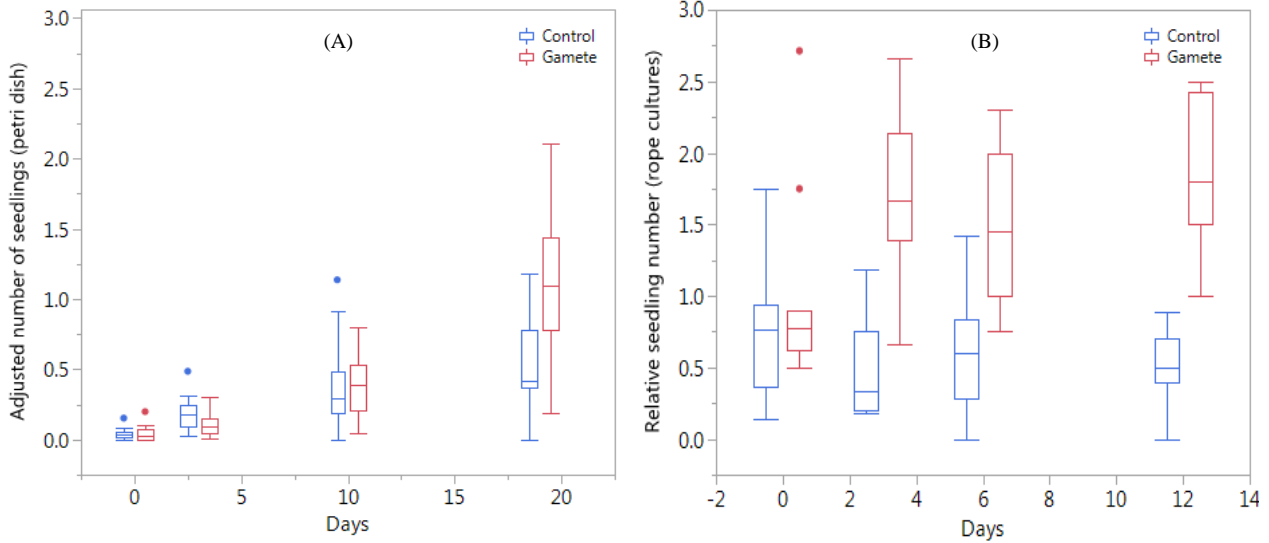
Figure 8.

276

277 3.3 Experiment 3: Including fertilization in hatchery

278 After 19 days in petri dish culture, the adjusted seedling number was 50.25 % higher (fig. 9A) with male  
 279 gametes solution added, compared to the control (two-tailed Student's t test:  $t(10)$ ,  $p < 0.0001$ ), whereas no  
 280 significant differences were found at the other days ( $p > 0.98$ ), except day 3, where the control group showed  
 281 a slightly higher number of seedlings ( $p = 0.0042$ ).

282 The effect of adding male gametes to the rope cultures (fig. 9B) was significant from day 3 (MANOVA:  $F_{1,19} =$   
 283  $121.445$ ,  $p < 0.0001$ ) with higher number of seedlings at all counting days (253.2 % at day 12). Yet, the effect  
 284 of time was insignificant on the number of seedlings (MANOVA:  $F_{3,17} = 0.5462$ ,  $p = 0.6573$ ), but caused a  
 285 significant interaction term of gamete addition \* time:  $F_{3,17} = 4.0842$ ,  $p = 0.0235$ , because of the higher  
 286 seedling count after day 0.



287  
288 Figure 9.

289 Discussion

290 The results of this study demonstrate effective methods and strategies in the pursuit of optimizing the  
 291 hatchery production of *P. palmata* by using vertical seeding tanks, consecutive use of sori packages and  
 292 agitation during the seeding phase. The strategy of using a secondary seeding inoculum of germinated  
 293 propagules, based on collecting the effluent spores, obviously increase the spore use efficiency. With  
 294 presented seeding system, 9 nets were spore-seeded, with an equivalent linear length of ~ 126 meter of  
 295 rope, using as little as 5 g FW sori, which result in an average of 9 seedling  $\text{cm}^{-1}$  after 32 days. The use of  
 296 spores for seeding substrates is prospective for larger scale cultivation (Browne 2001; Edwards 2007;  
 297 Werner and Dring 2011). The latter argued that an initial seeding density of 100 spores  $\text{cm}^{-1}$  is required to  
 298 obtain a final seedling density of ~ 6-8 seedlings  $\text{cm}^{-1}$  as a mortality rate of spores of 60-80% took place. On  
 299 top, only male gametophytes developed a thallus, representing 50% of the total amount of spores. The initial  
 300 spore density encountered in the present study (Exp. 1) on the nets was lower than 100 spores  $\text{cm}^{-1}$ ,  
 301 however, we estimated a spore germination success (reciprocal to mortality) of 50-80% on the nets similar to  
 302 what was achieved in a previous study (Le Gall et al., 2004). This indicates that the seeding and nursery  
 303 conditions presented here were good, though it might be overestimated, as we were not able to count the  
 304 dying spores. In contrast, we observed a germination success of only 8-14 % in petri dishes cultured in  
 305 stagnant seawater under the same conditions (unpublished work), which is in the range of previous report  
 306 (Edwards 2007; Edwards and Dring 2011). By using higher amounts of sori in the seeding phase of net, the  
 307 nets showed higher spore density after 3 days, but decreased to similar levels for all sori amount used, after  
 308 32 days of nursery, in line with previous findings (Edwards 2007; Werner and Dring 2011). In present study,  
 309 the average seedling density after 32 days of nursery was not significantly affected by the sori amounts  
 310 tested. Besides testing efficient ways to handle sori tissue for optimal spore yield, it is important that future  
 311 hatchery trials consider the sori-to-substrate density, as high spore density seem to even out during nursery,  
 312 thus the sori could have been used more efficient. A current hatchery protocol for *P. palmata*, found that a  
 313 sori-to-substrate ratio of 150 g FW sori  $84 \text{ m}^{-1}$  substrate was required to secure sufficient seedling density,

314 which converts to ~130 kg FW fronds to seed one long-line of 100 m (Werner and Dring 2011). In  
315 comparison, several kilometers of substrate can be seeded with motile zoospores of *Saccharina latissima* by  
316 using 150 g sori, due to the multifold number of biflagellate zoospores released within 1 hour and their  
317 capability of high dispersal (personal observation). We suggest that a lower amount of sori is sufficient for  
318 seeding, while considering the sori-to-substrate ratio.

319 Three times the amount of seeded substrate was produced by using three consecutive  
320 seeding periods, compared to the conventional 3-days seeding phase. The highest seedling density after 32  
321 days for nets seeding was found in the second (~8 seedlings cm<sup>-1</sup> and third seeding period (~10 seedlings cm<sup>-1</sup>).  
322 In the first seeding period (Day 0-3), our tank setup provided a spore-seeding efficiency of 16%, meaning  
323 that ~84 % of the released tetraspores were detained in the down-stream detaining tanks. The amount of  
324 detained spores was highest after the second seeding period (Day 3-6). Even 38 days after finalizing the last  
325 seeding period we observed red tetraspore aggregates in some of the spore-detaining tanks. This  
326 observation supports previous findings, where sori was observed to release for 21 days (Schmedes et al.  
327 2019) to 40 days (Wood 2018).

328 Several bottlenecks have been identified by using the current hatchery protocol for *P. palmata*  
329 for large-scale cultivation – a protocol where sori is placed above the cultivation substrates (1:1 areal  
330 coverage) in horizontal tanks, which impose a high sori requirement. Facing other challenges when hatching  
331 rhodophytes, such as seasonal variation in spore availability (Kain 1986; Le Gall et al. 2004), a relative low  
332 spore release yield (Edwards 2007), poor spore dispersal before settlement (Edwards and Dring 2011) and  
333 low survival of spores (Sanderson 2006; Werner and Dring 2011), all impose a low spore use efficiency and  
334 little control of seedling quality. Overall, an even spore dispersal and good seedling density was found in this  
335 study using a flow-through system and relative high aeration, agitating the water. Nevertheless, further  
336 investigations of the previous mentioned challenges are highly relevant to optimize before commercial  
337 hatcheries can be established. Hence, the recently developed GMA-method (Schmedes et al. 2019) to  
338 improve the spore efficiency for hatching *P. palmata* was investigated. This method was applied by  
339 dislodging and maceration of detained spores, which then germinated into propagules, which again were  
340 macerated to break aggregates of spores and tiny seedlings into single and small groups of spores and  
341 seedlings, before used as a secondary seeding inoculum. The level of water agitation of 2.5 L min<sup>-1</sup>  
342 dispersed the propagules, yet, did not compromise the establishment of a discoid reattachment on the ropes  
343 (Exp. 2). Results demonstrated that the reattachment was negatively affected by the biological age at  
344 dislodgement and amount of days in agitation, which adds knowledge to the extent of which macerated *P.*  
345 *palmata* propagules can be used as seeding inoculum. This is in agreement with findings in other red  
346 seaweed species, such as *Chondracanthus chamissoi* (Gigartinales), which forms a substrate reattachment  
347 by the production of secondary attachment discs (Sáez et al. 2008). The thallus fragments showed a  
348 decreasing reattachment probability over time (Fonck et al. 2008). Also, the red seaweed *Gelidium chilense*  
349 (Montagne) formed bundles of rhizoids in agitated water (Santelices and Varela 1994), while absent in  
350 *Gelidium coulteri* cultured in stagnant water conditions (Macler and West 1987). In comparison, a direct  
351 seeding method for cultivating the brown macroalgae species *S. latissima*, as a way to optimize hatchery  
352 duration and costs, is currently used by the Hortimare company, as a seeding technique for commercial



353 cultivation in Europe. Here, tiny germinated and activated sporophytes are applied to substrates and  
354 establish firm attachment with their developing haptera organs, with a potential benefit of using a glue  
355 (Kerrison et al. 2018). Whether the propagule seeding method of *P. palmata* would benefit by using glue as a  
356 means to maximize seedling density, is of high interest due to the high commercial value of the biomass.

357           The inclusion of a fertilization step proved to be highly relevant to increase the number of  
358 seedlings in the hatchery production, by releasing male gametes and adding this male gamete solution to the  
359 female gametophytes that are developing their trichogynes (Mine and Tatewaki 1994; Le Gall et al. 2004).  
360 This resulted in at least a doubling of the number of seedlings on spore-seeded rope and suggests that male  
361 gametes can be added 10-22 days after seeding substrates to increase the overall spore-use-efficiency of *P.*  
362 *palmata*.

363

#### 364 4. Conclusions

365 A new method is reported here to handle fertile sori of *P. palmata* for efficient release and dispersal of  
366 tetraspores using sori packages in vertical flow-through tanks. This achieves a sufficient seedling density ~9  
367 seedlings cm<sup>-1</sup> rope. Consecutive use of sori greatly improves the spore use efficiency and was found to  
368 have a positive effect on seeding quality, even for different sori amounts used in the sporulation phase. The  
369 spore inoculation success (net-seeding efficiency ~16%) was relative low, however, the ~84 % effluent  
370 spores was used in propagule inoculation by using the GMA-method. Hence, to increase the use of spores  
371 for efficient inoculation, the importance of addressing following parameters to further improve the spore-use  
372 efficiency for seeding *P. palmata* is needed. The following are of importance, i.e., tank typology, water  
373 motion for optimal spore dispersal and settlement, intermittent batch conditions to increase net seeding  
374 efficiency, substrate-to-volume density, sori position, and flow-through rate. It was demonstrated that effluent  
375 spores from the SDTs were suited as a secondary seeding inoculum, using the Germinate-Macerate-Agitate  
376 seeding method (Schmedes et al. 2019).

377

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446

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454 Compliance with Ethical Standards

455 Informed consent of the document with no conflict of interest. We did not conduct research on human or  
456 animals.

457

458 Figure 1. A: Parallel flow-through setup of conical seeding tanks (30 L) used for experiment 1. B: The sori  
459 packages were fixed centrally above the net spirals and 2 cm below water surface. Effluent spores were  
460 detained in spore-detaining tanks (SDTs). C: Aeration ( $1.2 \text{ L min}^{-1}$ ) from the bottom provided hemispherical  
461 water circulation and dispersal of released spores.

462 Figure 2. A: Fertile tetrasporophytic fronds were cleaned and the presence of dark-red sporangia were  
463 validated (B). C: Sori packages (5, 10, or 15 g FW) were prepared by placing the sori between two layers of  
464 green plastic mesh ( $n=3$ ).

465 Figure 3. A: Dark-red aggregates of spores on the bottom of spore-detaining tanks (STDs) accumulated  
466 during sporulation. B: A well-mixed spore solution was obtained by dislodging the aggregates and  
467 macerating them. C: The number spores were counted in five random fields (each of  $0.0035 \text{ mm}^2$ ) and used  
468 as a basis to estimate the total amount of detained spores.

469 Figure 4. A: Detained and macerated spores of *P. palmata* at the bottom of a 5 L Plexiglas tank. B: Plexiglas  
470 tank after dislodgement of propagules on the left side of the tank. C: Propagule mix after dislodgement and  
471 maceration. The solution was inspected before (D) and after (E) the maceration treatment. F: Single  
472 individual seedling displaying hair-like proliferations from the basal disc area. Scale bar represent 500  $\mu\text{m}$ .

473 Figure 5. A: Fertile male gametophytes inspected by stereomicroscopy. B: cross-section microscopy was  
474 used to verify reproductive appearance of the males. C, D: Spherical male gamete released in a solution.

475 Figure 6. The number of spores (A-C) and seedlings (D-F) attached to the nets and the spore germination  
476 percentage (G-I). 4 cm net pieces were counted on day 3, 19 and 32 after each of the three consecutive  
477 periods (Day 0-3, Day 3-6, Day 6-9) with the use of different sori amount (5, 10, 15 g). Data is presented as  
478 mean $\pm$ SE,  $n=3$ .

479 Figure 7. A-I: The extrapolated number of spores attached to the nets and (D-F) the total number of detained  
480 spores collected in the spore-detaining tanks (SDTs) as a function of three consecutive seeding periods and  
481 the use of three different amount of sori (5, 10, 15 g FW). G-I: Net seeding efficiency, calculated from  
482 equation 1. Data is presented as mean $\pm$ SE,  $n=3$ .

483 Figure 8. Attached spores (A-B) and seedlings (C-D) on rope pieces seeded with macerated propagule  
484 solution at day 3 and 10 and as a function of age at dislodgement (day 27-29 vs. day 37-39) and agitation  
485 period (day 0-2 vs. day 10-12). Data is presented as outlier box plots (1<sup>st</sup>, 3<sup>rd</sup> quartile whisker) based on  
486 three subpart counts on each of ten pieces of rope ( $n=10$ ,  $N=30$ ).

487 Figure 9. A: The normalized seedling count in petri dish cultures ( $n=10$ ) of macerated *P. palmata* propagules  
488 as an effect of adding male gametes. B: The normalized seedling count on spore-seeded rope cultures  
489 ( $n=11$ ) as an effect of adding male gametes. Data is presented as outlier box plots (1<sup>st</sup>, 3<sup>rd</sup> quartile whisker).