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1 Effect of essential oil- and iodine 2 treatments on the bacterial microbiota of 3 the brown alga *Ectocarpus siliculosus*

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9 ABSTRACT

10 Macroalgae live in tight association with bacterial communities, which impact most
11 aspects of their biology. Clean, ideally axenic, algal starting material is required to
12 control and study these interactions. Antibiotics are routinely used to generate clean
13 tissue; however, bacterial resistance to antibiotics is increasingly widespread and
14 sometimes related to the emergence of potentially pathogenic, multi-resistant strains.
15 In this study, we explore the suitability of two alternative treatments for use with algal
16 cultures: essential oils (EOs; thyme, oregano, and eucalyptus) and povidone-iodine.
17 The impact of these treatments on bacterial communities was assessed by bacterial
18 cell counts, inhibition diameter experiments, and 16S-metabarcoding. Our data show
19 that thyme and oregano essential oils (50% solution in DMSO, 15h incubation)
20 efficiently reduced the bacterial load of algae without introducing compositional biases,
21 but they did not eliminate all bacteria. Povidone-iodine (2% and 5% solution in artificial
22 seawater, 10min incubation) both reduced and changed the alga-associated bacterial
23 community, similar to the antibiotic treatment. The proposed EO- and povidone-iodine
24 protocols are thus promising alternatives when only a reduction of bacterial abundance
25 is necessary and where the phenomena of antibiotic resistance are likely to arise.

26 **Keywords:** Antibiotics, Essential oils, Povidone-iodine, Brown algae, Microbiome,
27 Metabarcoding

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28 INTRODUCTION

29 The biology of macroalgae can only be fully understood by taking into account
30 the interactions with their microbiomes, which impact their health, performance and
31 resistance to stress (Goecke et al. 2010). Together, both components form a singular
32 functional entity, the holobiont (Margulis 1991). Studying holobiont systems implies
33 studying the individual components of the holobiont, their diversity, their activities and
34 the (chemical) interactions between them (Goecke et al. 2010; Wahl et al. 2012;
35 Hollants et al. 2013; Dittami et al. 2020). Elucidating these interactions requires
36 controlled algal-bacterial co-cultivation experiments to test hypotheses about the
37 functions of specific microbes. This, in turn, equally depends on the isolation of
38 bacterial strains and the availability of aposymbiotic algal starting material, *i.e.* algae
39 without the presence of any symbionts.

40 Antibiotics are routinely used to generate such aposymbiotic cultures, yet
41 bacterial resistance to antibiotics is increasingly widespread and sometimes related
42 to the emergence of potentially pathogenic, multi-resistant strains (Fair and Tor 2014).
43 Spices and essential oils (EOs) are promising alternatives to antibiotics and have been
44 used as antiseptics since antiquity (McCulloch 1936). However, it was only towards
45 the end of the 19th century that Chamberland (1887) first systematically evaluated the
46 antibacterial properties of several EOs. Today, numerous studies assessing the
47 efficiency of EOs against bacteria are available (e.g. Deans and Ritchie 1987; Burt
48 2004; Bakkali et al. 2008) including one in marine bacteria (Mousavi et al. 2011), but
49 none so far targeting algae-associated microbiomes.

50 A second alternative to antibiotics may be iodine-based treatments. Berkelman
51 et al. (1982) have shown that diluted solutions of povidone-iodine have antibacterial
52 effects on *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas cepacia*,
53 and *Streptococcus mitis*. Furthermore, povidone-iodine may be active against
54 anaerobic or sporulated organisms, moulds, protozoans, and viruses (Zamora 1986).
55 Kerrison et al. (2016) have obtained promising results using potassium iodine
56 solutions to remove parts of the microbiota of red- and green algae. However, the
57 effect of povidone-iodine on brown algae and their associated microbiota may be
58 different as some brown algae are known to naturally accumulate high concentrations
59 of iodide in their cell wall. The algae use this iodine for defence reactions (Küpper et

60 al. 2008; La Barre et al. 2010), and brown algae-associated microbes may have
61 developed higher tolerance levels for such treatments.

62 In this study, we examined the suitability of three different EO treatments
63 (thyme, oregano, peppermint eucalyptus) as well as one povidone-iodine treatment to
64 reduce and control the microbiome associated with the filamentous brown alga
65 *Ectocarpus siliculosus*. *E. siliculosus* has been established as a genomic model for
66 the brown algal lineage (Cock et al. 2010), but the genus *Ectocarpus* has recently also
67 gained in importance for the study of brown algal-bacterial interactions (Dittami et al.
68 2016; Tapia et al. 2016; KleinJan et al. 2017; Burgunter-Delamare et al. 2020). Our
69 data show that all tested EOs efficiently reduced the bacterial load of algae without
70 introducing compositional biases, but they did not eliminate all bacteria. Povidone-
71 iodine treatments, just as the antibiotics, both reduced and changed the algae-
72 associated bacterial community.

73 MATERIALS & METHODS

74 Algal cultures

75 *Ectocarpus siliculosus* strain Ec32 (CCAP accession 1310/04, isolated from San
76 Juan de Marcona, Peru) was cultivated in 90 mm Petri dishes at 13°C under a 12h/12h
77 day-night cycle and 40 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ irradiance provided by daylight-type
78 fluorescent tubes. The culture medium was composed of autoclaved natural seawater
79 (NSW) enriched with Provasoli nutrients (PES; Provasoli and Carlucci 1974).

80 Essential oil treatments

81 We tested the effect of three EOs known for their antibacterial properties on the
82 *E. siliculosus* bacterial microbiome: **thyme** (*Thymus vulgaris*), **oregano** (*Origanum*
83 *vulgare*), **peppermint eucalyptus** (*Eucalyptus dives piperitoniferum*) (Nelson 1997;
84 Dorman and Deans 2000; Burt and Reinders 2003; De Billerbeck 2007; Kaloustian et
85 al. 2008; Da Silva 2010; Amrouni et al. 2014). The EOs were purchased from
86 AromaZone (Paris, France), and were rated as 100% pure.

87 EOs are, however, natural products and as such, their complex chemical
88 composition is subject to variation. For this reason, the composition of the EOs used
89 in our experiments was determined by GC/MS analyses based on a protocol adapted

90 from Habbadi et al. (2017). Ten μL of each EO was diluted in 990 μL of pure hexane
91 (Supelco Analytical, Bellefonte, PA, USA) and 1 μL of the solution was injected in an
92 Agilent GC7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA)
93 equipped with a DB-5MS capillary column (30m \times 0.25 mm i.d., film thickness 0.25 μm ,
94 Agilent Technologies) and coupled to a model 5975C mass selective detector (positive
95 mode). Pure hexane was run as blank. This experiment was carried out in triplicate.
96 The oven temperature was initially maintained at 50°C and then increased to 300°C at
97 a rate of 7°C.min⁻¹. The injector temperature was 290°C. The carrier gas was purified
98 helium, with a flow rate of 1 mL.min⁻¹, and the split ratio was 60:1. Mass spectra were
99 obtained in EI mode at 70 eV ionisation energy and the mass range was from m/z 35
100 to 400. For each compound, the Kovats retention index (RI) was calculated relative to
101 a standard mix of n-alkanes between C7 and C40 (Sigma-Aldrich, St. Louis, MO, USA),
102 which was analysed under identical conditions. Constituents were identified by
103 comparing the RI and MS spectra to those reported in the literature (Adams 2007) and
104 by comparison with the NIST reference database. These analyses were performed at
105 the Corsaire-Metabomer platform at the Station Biologique de Roscoff.

106 Algal filaments were treated with EOs by EO diffusion on Zobell plates (tryptone
107 5 g.L⁻¹, yeast extract 1 g.L⁻¹, sterile seawater 80%, milliQ water 20%, agar 15 g.L⁻¹),
108 similar to an antibiogram in two rounds: the first round consisted of testing several
109 dilutions of the separate EOs in DMSO (Sigma-Aldrich, St. Louis, MO, USA) as well as
110 combinations of different EOs. In the second round, we focussed on the most
111 promising treatments, and an assessment of the microbial composition was added.
112 Under a laminar flow hood, sterile filter paper discs (diameter 10 mm, Whatman, GE
113 Healthcare, Buckinghamshire, UK) were soaked with 15 μL of EO solution and then
114 placed in the centre of a 90 mm Zobell plate. We included pure DMSO, NSW, and olive
115 oil as controls. *E. siliculosus* filaments were placed at 2 cm of the disc limit and plates
116 were incubated for 15 h at 13°C. Next, we briefly incubated the filaments in 25 mL
117 NSW to remove traces of the treatment and left them to recover for two weeks in PES
118 medium at 13°C. All experiments were carried out in triplicate. Treatments were
119 considered lethal when algal filaments entirely lost their pigmentation and no growth
120 was observed during the recovery period.

121 Microbial colonisation of the algal surface was determined both at the start of
122 the experiment and after the two-week recovery period. Bacterial cell counts were

123 performed by phase-contrast microscopy (Olympus BX60 microscope, 1.3-PH3
124 immersion objective, at 1000X magnification). The total number of bacteria was
125 determined over a distance of 100 μm and five independent counts were averaged per
126 biological replicate.

127 Povidone-iodine treatments

128 Povidone-iodine treatments were carried out by immersion of *E. siliculosus*
129 filaments in povidone-iodine solutions as described by Kerrison et al. (2016). Again, a
130 first round of experiments was carried out to determine the most efficient
131 concentrations and incubation times: solutions at 100 $\text{mg}\cdot\text{mL}^{-1}$ (Bétadine dermique
132 10%, Meda Manufacturing, Mérignac, France) and dilutions at 75 $\text{mg}\cdot\text{mL}^{-1}$, 10 $\text{mg}\cdot\text{mL}^{-1}$,
133 5 $\text{mg}\cdot\text{mL}^{-1}$, 1.33 $\text{mg}\cdot\text{mL}^{-1}$ and 0.67 $\text{mg}\cdot\text{mL}^{-1}$ were tested with incubation times of 30
134 sec, 1 min, 2 min, and 10 min (Berkelman et al. 1982; Kerrison et al. 2016). Each algal
135 filament was placed in a sterile 1.5 ml Eppendorf tube, incubated with 1 ml of iodine
136 solution for the given duration and washed with NSW before leaving the alga to recover
137 for two weeks in PES medium. The bacterial abundance on the algal surfaces was
138 examined by microscopy both at the start of the experiment and after recovery, as
139 described above.

140 The second round of experiments was then carried out focusing on one
141 promising experimental condition (10 min treatment, 1/20 dilution), adding notably an
142 assessment of the microbial community composition.

143 Antibiotic treatments

144 We included a standard antibiotic treatment parallel to the EO- and iodine
145 treatments (KleinJan et al. 2017) as a comparison to the new alternative methods. For
146 this treatment, filaments of *E. siliculosus* were incubated in 90 mm Petri dishes with 25
147 mL of antibiotic solution (penicillin G 45 $\mu\text{g}\cdot\text{mL}^{-1}$, streptomycin 22.5 $\mu\text{g}\cdot\text{mL}^{-1}$,
148 chloramphenicol 4.5 $\mu\text{g}\cdot\text{mL}^{-1}$ dissolved in NSW) for four days. The algae were left to
149 recover for three days in 25 mL of NSW and then re-treated for four days with 25 mL
150 of antibiotic solution. This was followed by another recovery period of 2 weeks in PES
151 medium. Bacterial cells on algal surfaces were counted before the experiments and
152 after recovery, as described above.

153 Determination of inhibition diameters

154 In addition to examining the treatment effect on bacteria in algal cultures, we
155 determined **inhibition diameters** as a direct measure of the treatment efficiency. *E.*
156 *siliculosus* filaments cultivated in PES were ground in a mortar with one mL of NSW.
157 Fifty µL of the obtained suspension was then plated on Zobell plates. Sterile paper filter
158 discs (10 mm, Whatman) were each soaked with 15 µL of the EO and iodine treatment
159 solutions described above, and one disc was placed in the centre of each inoculated
160 plate. Plates were cultivated for one week, which was followed by measurement of
161 inhibition diameter. Results were separated according to two levels of activities for the
162 discs soaked with the EO solutions and povidone-iodine solutions: resistant (ID < 12
163 mm) or susceptible (ID > 24 mm) (adapted from Ponce et al. 2003). Contrary to
164 classical determinations of inhibition diameters which usually focus on one strain of
165 bacteria, results from these experiments apply to the entire community of bacteria
166 associated with *E. siliculosus* at the time of the experiments.

167 Impact of treatments on microbial community

168 We determined the bacterial community composition associated with algal
169 cultures by 16S metabarcoding analyses before selected treatments (50% EO, 1/20
170 dilution of povidone-iodine for 10 min) as well as after the recovery period. For each
171 sample, about 20mg of freeze-dried algae was ground (2x45 sec at 30 Hz) with a
172 TissueLyser II (Qiagen, Hilden, Germany). DNA was extracted using the DNeasy Plant
173 Mini Kit (Qiagen) following the manufacturer's protocol. A mock community, comprising
174 a mix of DNA from 26 cultivated bacterial strains (Thomas et al. 2019), as well as a
175 negative control, were run and treated in parallel to the DNA extracts. For all of these
176 samples we amplified the V3 and V4 regions of the 16S rDNA gene following the
177 standard Illumina protocol for metabarcoding (Illumina 2013) and using the Q5® High-
178 Fidelity PCR Kit (New England BioLabs, MA, USA), the AMPure XP for PCR
179 Purification Kit (Beckman Coulter, Brea, CA, USA), and the Nextera XT DNA Library
180 Preparation Kit (Illumina, San Diego, CA, USA). Libraries were quantified with a Qubit
181 High-Sensitivity dsDNA Assay (Life Technologies, Carlsbad, CA, USA) and mean
182 fragment size was determined using a Bioanalyzer 2100 system (Agilent Technologies,
183 Santa Clara, CA, USA). An equimolar pool of all samples was generated at a
184 concentration of 4nM, diluted to 5 pM, spiked with 20% PhiX (Illumina) and sequenced

185 on an Illumina MiSeq sequencer on the Genomer platform (Station Biologique de
186 Roscoff) using a MiSeq v3 kit (2x300bp, paired-end).

187 The obtained reads were cleaned using Trimmomatic version 0.38 (Bolger et al.
188 2014), assembled using Pandaseq v2.9 (Masella et al. 2012) and then analysed with
189 Mothur 1.40.3 according to the MiSeq standard operating procedures developed by
190 Kozich et al. (2013). Briefly, we aligned the sequences with the Silva_SEED database
191 version 132 and removed non-aligning sequences, chimeric sequences (identified by
192 vsearch), organellar sequences (identified by RDP classifier) and sequences that were
193 represented only once in the dataset (singletons). The remaining sequences were then
194 clustered into operational taxonomic units (OTUs) at a 97% identity level. OTUs that
195 were more abundant in the blank samples compared to all other samples as well as
196 rare OTUs (<5 reads in all samples taken together) were removed from the dataset.
197 Finally, the OTU matrix was sub-sampled to avoid biases in the subsequent analyses.

198 [Statistical tests](#)

199 We compared bacterial counts and inhibition diameters across conditions using
200 an ANOVA test followed by a Tukey HSD test using the Multcomp package of the R
201 software (version 1.0.44) and a p-value cutoff of 0.05. The normality of the input data
202 was verified with a Shapiro-Wilk test, but slight deviations from a normal distribution
203 were tolerated (Underwood 1981).

204 Principal component analyses (PCAs) were carried out on the bacterial
205 sequence abundance data using the DESeq2 package (Love et al. 2014). This
206 package was also used to determine OTUs that differed significantly in relative
207 abundance between treatments allowing for a false discovery rate of 5%. Binomial
208 tests followed by a Benjamini and Hochberg correction (Benjamini and Hochberg 1995)
209 were carried out to determine the overrepresented families among the impacted OTUs.

210 **RESULTS**

211 [Essential oil composition](#)

212 GC-MS analyses of the thyme, oregano, and eucalyptus EOs led to the
213 identification of 34 different chemical compounds (**Table 1**), mainly phenols,
214 monoterpenols, and monoterpenes. The EO of *Thymus vulgaris* was mainly composed
215 of thymol (57.44%), γ -terpinene (20.88%), p-cymene (5.41%), and carvacrol (4.64%).

216 The major constituents of *Eucalyptus dives piperitoniferum* EO were piperitone
217 (63.74%), α -phellandrene (12.9%), and terpinen-4-ol (4.45 %). The EO of *Origanum*
218 *vulgare* was mainly composed of carvacrol (78.01%), p-cymene (7.82%), γ -terpinene
219 (4.31%), and thymol (4%). These chemical compositions are consistent with the
220 literature (Gilles et al. 2010; Amrouni et al. 2014; Habbadi et al. 2017).

221 Antimicrobial effects of EOs and povidone-iodine in cultures

222 The number of bacteria on the algal surface at the start of the experiments
223 compared to the number of bacteria on the algal surface after the treatments and the
224 2-week recovery are shown in **Table 2**. For the **EO treatments**, the olive oil and DMSO
225 control showed no antibacterial effect. All combinations of different EOs were lethal for
226 the algae at the concentrations tested. The remaining individual EOs exhibited various
227 levels of antimicrobial activity with the 50% solutions being the most efficient.
228 Concordant results were also obtained in the second round of experiments (**Table 3**),
229 although the effect of eucalyptus was no longer statistically significant. The inhibition
230 experiments with ground cultures revealed that only the thyme and oregano EOs
231 resulted in inhibition diameters (IDs) > 25 mm (**Table 4**). For the eucalyptus treatments,
232 IDs were below the defined threshold for at least one of the bacteria present in the
233 alga-associated microbiota.

234 The stock solution of **povidone-iodine** was lethal for the algae, but the 1/20 and
235 1/50 diluted solutions, combined with a treatment time of 10 minutes, proved to be
236 efficient in both experiments (**Table 2, Table 3**). In the inhibition diameter experiments,
237 only the 75 mg.mL⁻¹ solution of povidone-iodine resulted in an inhibition diameter > 25
238 mm (**Table 4**). For the other treatments, including the **antibiotic treatment**, inhibition
239 diameters were below the defined threshold for at least one of the bacteria present in
240 the alga-associated microbiota.

241 **In algal cultures**, unlike in the inhibition diameter experiments, the efficiency of
242 all EO and povidone-iodine treatments was low compared to that of the treatment with
243 antibiotic-solution, which generally resulted in two- to ten-fold lower bacterial loads
244 after recovery (**Table 2, Table 3**).

245 Effect of treatments on bacterial community composition

246 16S metabarcoding analyses were carried out for all control samples as well as
247 for those treated with the 20-fold dilution of povidone-iodine, the 50% EO solutions or
248 the antibiotics. The sequences obtained corresponded predominantly to
249 *Alphaproteobacteria* (59% of reads), followed by *Bacteroidetes* (28.3% of reads),
250 *Gammaproteobacteria* (4.6% of reads), and *Actinobacteria* (2.2% of the reads across
251 all experiments; **Fig. 1**). A total of 9 818 OTUs were identified in the dataset.

252 For the EO treatments, DESeq2 analyses revealed no significant effect on the
253 microbial community composition as confirmed by the PCA plots (**Fig. 2a**). For the
254 povidone-iodine treatments, the PCA showed a clear separation of controls kept in
255 NSW and treated samples for the iodine treatment (**Fig. 2b**). A total of 252 OTUs were
256 found to differ significantly (adjusted $p < 0.05$) in relative abundance between the treated
257 and non-treated samples (69 OTUs decreased and 183 increased in treated samples;
258 Supplementary data **Table S1**). The taxonomic affiliation of those OTUs is shown in
259 **Table 5**. Among the OTUs that were negatively impacted by the povidone-iodine
260 treatment and that were significantly overrepresented (adjusted $p < 0.05$) are: an
261 unclassified family of *Acidicribiia*, an unclassified family of *Microtrichales*, an
262 unclassified family of *Actinobacteria*, as well as the *Saprospiraceae* and
263 *Rhodobacteraceae* families. Among the OTUs that increased in relative abundance in
264 response to the povidone-iodine treatments and that were significantly
265 overrepresented (adjusted $p < 0.05$) are: the *Cyclobacteriaceae*, *Hyphomonadaceae*,
266 *Sphingomonadaceae*, *Alteromonadaceae*, *Haliaceae*, and *Pseudohongiellaceae*
267 families.

268 For the antibiotic treatments, due to their high efficiency, no visible bands were
269 obtained during PCR amplification for metabarcoding. Library preparation was
270 nevertheless carried out, but only 10 reads remained after cleaning. These reads were
271 associated with the class of *Alphaproteobacteria*, notably the *Rhizobiaceae* and
272 *Rhodobacteraceae* families and the *Marinobacter* genus.

273 DISCUSSION

274 Antibiotic treatments are commonly used to obtain clean algal cultures, yet
275 bacterial resistance to antibiotics is increasingly widespread. Sometimes it is related to
276 the emergence of pathogenic, multi-resistant bacterial strains. Thus, especially after
277 long treatments, resistant strains may proliferate without control from the remaining
278 microbiome, sometimes by far exceeding bacterial concentrations found in a healthy
279 microbiome (personal data). Ethanol has been proposed as one alternative treatment
280 to clean kelp species, e.g. in *Ecklonia radiata*, where a short bath in a 70% ethanol
281 solution followed by sterile deionised water showed promising results (Lawlor et al.
282 1991). In much the same way, the surfaces of the wrack *Fucus serratus* and the red
283 alga *Palmaria palmata* surfaces can be cleaned efficiently with a mixture of ethanol
284 (40–50%) and sodium hypochlorite (1%) (Kientz et al. 2011). Unfortunately, such
285 surface sterilisation methods are not suitable for small filamentous algae such as
286 *Ectocarpus*. When *Ectocarpus* filaments come in to contact with 70% ethanol or
287 bleach, even for less than a second, this results in immediate loss of pigmentation and
288 cell death. Therefore, we sought to test two other alternative treatments, EOs and
289 povidone-iodine, to reduce the microbiota associated with the brown alga *E.*
290 *siliculosus*, and compared the results with the standard antibiotic treatment routinely
291 used in our laboratory. Moreover, unlike in previous studies that focused exclusively
292 on the direct impact of treatments on the number of bacteria on algal surfaces, our
293 study also examined the taxonomic composition of the microbiome after recovery.

294 Essential oils inhibit the growth of the complete spectrum of *Ectocarpus*-associated 295 bacteria

296 Our data show that the tested EO treatments significantly reduce the number of
297 bacteria associated with *E. siliculosus* even after two weeks of recovery. This is in line
298 with data published by Mousavi et al. (2011), who observed a strong impact of a
299 combination of four EOs on several bacterial isolates, both marine and terrestrial. A
300 key point that has not been previously demonstrated is that this reduction occurred
301 without significant change in the relative bacterial community composition. Indeed,
302 EOs contain several molecules such as p-cymene, β -phellandrene, terpinolene,
303 terpinen-4-ol, piperitone, carvacrol, and thymol, which have been shown to have an
304 antibacterial effect on a wide range of bacteria (Lambert et al. 2001; Carson et al. 2002,

305 2006; Eftekhar et al. 2005; Bakkali et al. 2008; Mora et al. 2011; Marchese et al. 2016,
306 2017). The fact that thyme and oregano were more efficient than eucalyptus in our
307 experiments could be due to their higher concentration of linalool. This compound has
308 been shown to have a synergic effect when combined with thymol and carvacrol
309 molecules (the principal components of thyme and oregano EOs) (Bassolé et al. 2010;
310 Herman et al. 2016). Both thymol and carvacrol target the bacterial cell membrane.
311 Carvacrol changes membrane permeability for essential cations like H⁺ and K⁺, leading
312 to leakage and cell death (Ultee et al. 1999), and thymol inserts itself in the lipid
313 membrane, changing its morphology and disrupting the surface elasticity (Ferreira et
314 al. 2016).

315 Furthermore, EOs contain several other potentially antimicrobial molecules.
316 Due to this complex composition, the overall antibacterial activity of EOs is likely
317 caused by a broad spectrum of mechanisms of action (Burt 2004; Bakkali et al. 2008),
318 contrary to antibiotics. For this reason, it is expected that bacteria might rarely develop
319 resistance mechanism for EOs. On the downside, host tolerance of high
320 concentrations of EOs may also be limited, as illustrated by the lethal effect on algal
321 hosts observed for the EO mixtures described herein.

322 [Povidone-iodine treatments induce microbial community shifts](#)

323 Povidone-iodine at low concentrations was also an efficient inhibitor of overall
324 bacterial growth. The active compound in povidone-iodine is 'free' iodine (McDonnell
325 and Russell 1999). Povidone-iodine is an iodophor, a complex of iodine and a
326 solubilising carrier (poly-vinyl-pyrrolidone, PVP), which acts as a reservoir of free
327 iodine. The free iodine levels are dependent on the concentration of the povidone-
328 iodine solution. The content of non-complexed free iodine increases as the dilution
329 increases, reaching a maximum value at about 0.1% final concentration (*i.e.* a 1/100
330 dilution), but then decreases again with further dilution (Rackur 1985). The PVP
331 component increases the antimicrobial efficiency of iodine by delivering the iodine
332 directly to the bacterial cell surface as a result of its affinity to cell membranes (Zamora
333 1986).

334 Bacterial resistance to povidone-iodine is rare in a medical context (Houang et
335 al. 1976), probably because its principle of action, the rapid oxidation of amino acids
336 and nucleic acids in biological structures (Kanagalingam et al. 2015), is hard to

337 counteract. However, iodine is also known to accumulate naturally in brown algae,
338 which emit volatile short-lived organo-iodines and molecular iodine as part of their
339 molecular defence repertoire (Leblanc et al. 2006; Küpper et al. 2008). It is therefore
340 likely that microbes in long-lasting associations with brown algae have at least a basic
341 level of resistance against iodine-based defences. In fact, some marine bacteria
342 associated with algae even have their own iodine metabolism or iodine uptake
343 mechanisms (Amachi et al. 2007; Fournier et al. 2014; Barbeyron et al. 2016). For
344 instance, *Zobellia galactanivorans* (*Flavobacteria*) efficiently degrades brown algal cell
345 walls and has been suggested to cope with reactive oxygen species and the massive
346 amounts of liberated iodine via the activity of a vanadium-dependent iodoperoxidase
347 (Fournier et al. 2014; Barbeyron et al. 2016). The presence of such iodine-specialised
348 marine bacteria may explain why, unlike EOs, iodine treatments resulted in a specific
349 shift in microbial community composition after application.

350 Among the 69 OTUs significantly reduced by the povidone-iodine treatment,
351 several belonged to the *Actinobacteria*, which are known to be affected by this
352 molecule (Lachapelle et al. 2013). Furthermore, *Actinobacteria*, *Chitinophagales* and
353 *Rhodobacteraceae* were found only among the negatively impacted OTUs. On the
354 other hand, *Cytophagales*, *Hyphomonadaceae*, *Alteromonadaceae*, *Haliaceae*, and
355 *Oceanospirillales* comprised many OTUs that increased in relative abundance in
356 response to the povidone-iodine treatments. An increase in relative abundance does
357 not necessarily indicate an increase in absolute abundance as global bacterial cell
358 counts decreased in response to the treatments; however, these taxa are likely to have
359 more widespread resistance mechanisms to iodine and may benefit from the creation
360 of a new niche as other bacteria in the community decline. A key question for the future
361 is to understand how these bacteria tolerate iodine and if this tolerance correlates in
362 any way with the iodine metabolism of the algal host.

363 CONCLUSION AND OUTLOOK

364 While antibiotic treatments are currently the most efficient way of eliminating
365 algal-associated microbiota and cannot be replaced by any of the tested alternative
366 treatments in the near future, both EOs and povidone-iodine offer promising
367 alternatives when only a reduction of bacterial abundance is sought and where the

368 phenomena of antibiotic resistance are likely to become an issue. Notably, this is the
369 case in aquaculture, the use of antibiotics may disrupt the equilibrium between bacteria
370 and lead to the proliferation of resistant bacterial strains, including opportunistic
371 pathogens (Watts et al. 2017). In seaweed aquaculture, the notion of controlling or
372 manipulating the microbiome is not yet widespread, but it is known that microbiota
373 impact algal fitness (Goecke et al. 2010; Wahl et al. 2012) and even the chemical
374 properties of the algae (Burgunter-Delamare et al. 2020). In the hatchery (closed)
375 stages of seaweed aquaculture, both EOs and iodine treatments could potentially be
376 used as one way of modifying the microbiome, possibly in combination with probiotics
377 (Suvega and Arunkumar 2019). The protocols proposed here may prove useful in this
378 context as they are more likely to be tolerated - even by small and filamentous algae.
379 Moreover, knowledge on the compositional biases introduced by the treatments may
380 help orient potential users towards either one of the proposed treatments depending
381 on their aims.

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Table 1 - Chemical composition of *Origanum vulgare*, *Thymus vulgaris*, and *Eucalyptus dives piperitoniferum* essential oils. Compounds that represent more than 1% of the total peak area are indicated in italic. RI = retention index.

RI	Compounds	% peak area		
		<i>Origanum vulgare</i>	<i>Thymus vulgaris</i>	<i>Eucalyptus dives piperitoniferum</i>
927	<i>α-thujene</i>	0.22	–	1.28
935	α -pinene	0.59	0.16	–
952	Camphene	0.11	0.26	–
980	β -pinene	0.18	–	–
989	β -myrcene	0.50	0.16	0.55
1008	<i>α-phellandrene</i>	–	–	12.90
1019	α -terpinene	0.51	0.24	0.84
1026	<i>p-cymene</i>	7.82	5.41	4.05
1033	<i>β-phellandrene</i>	–	–	1.76
1035	Eucalyptol	0.06	0.80	–
1046	3-carene	–	–	0.25
1060	γ -terpinene	4.31	20.88	0.60
1087	<i>Terpinolene</i>	–	–	1.73
1100	<i>Linalool</i>	1.29	1.53	0.58
1127	Menth-2-en-1-ol <cis-p->	–	–	0.22
1139	Trans-verbenol	0.23	–	–
1151	<i>Camphor</i>	–	1.43	–
1176	<i>Borneol</i>	–	1.41	–
1184	<i>Terpinen-4-ol</i>	–	0.67	4.45
1186	Thujone	–	–	0.28
1197	<i>α-terpineol</i>	–	0.24	1.12
1239	Thymol methyl ether	–	0.39	–
1257	<i>Piperitone</i>	–	–	63.74
1291	<i>Thymol</i>	4.00	57.44	–
1300	<i>Carvacrol</i>	78.01	4.64	–
1372	4,6-di-tert-butylresorcinol	–	0.18	–
1427	<i>β-caryophyllene</i>	1.61	2.11	0.77
1467	Naphthalene	–	0.36	0.46
1497	Viridiflorene	–	–	0.74
1502	<i>Elixene</i>	–	–	2.87
1591	<i>Caryophyllene oxide</i>	0.50	1.45	–

Table 2 - Ratio after treatment / before treatment of the number of bacteria on the algal surface. (\pm standard deviation, n=3). NSW: natural sea water, ATB: antibiotics, -: not tested, +++: bacterial proliferation. *: Significant results in comparison with the control (p -value<0.05).

		Stock solution	Dilution 3/4	Dilution 1/2	Dilution 1/10	Dilution 1/20	Dilution 1/50	Dilution 1/100	NSW
Essential oil	DMSO	1.12 \pm 0.28	-	-	-	-	-	-	-
	Olive oil	+++	-	+++	+++	-	-	-	-
	Eucalyptus	0.38 \pm 0.15 *	-	0.30 \pm 0.12 *	-	-	3.41	-	-
	Oregano	0.49 \pm 0.10 *	-	0.35 \pm 0.10 *	-	-	0.79 \pm 0.24	-	-
	Thyme	1.10 \pm 0.17	-	0.35 \pm 0.12 *	-	-	0.44 \pm 0.29 *	-	-
	Thyme + oregano	algal death	-	algal death	algal death	-	-	-	-
	Thyme + eucalyptus	algal death	-	algal death	algal death	-	-	-	-
	Eucalyptus + oregano	algal death	-	algal death	algal death	-	-	-	-
	Eucalyptus + oregano + thyme	algal death	-	algal death	algal death	-	-	-	-
Antibiotics	NSW	-	-	-	-	-	-	-	0.60 \pm 0.22
	ATB	0.06 \pm 0.05 *	-	-	-	-	-	-	-
Iodine	NSW	-	-	-	-	-	-	-	0.60 \pm 0.22
	30sec	dead	0.55 \pm 0.06	-	-	-	0.33 \pm 0.01 *	0.62 \pm 0.15	0.54 \pm 0.14
	1min	dead	0.46 \pm 0.02 *	-	-	-	0.43 \pm 0.11 *	0.87 \pm 0.02	1.35 \pm 0.21
	2min	dead	0.89 \pm 0.09	-	-	-	0.29 \pm 0.16 *	1.01 \pm 0.03	17.27 \pm 0.12
	10min	-	-	-	0.51 \pm 0.11	0.34 \pm 0.15 *	0.17 \pm 0.08 *	-	0.86 \pm 0.08

Table 3 – Average number of bacteria after the treatments and 2 weeks of recovery. (\pm standard deviation, n=3). *: Significant results compared to controls (DMSO/NSW). NSW: natural sea water.

	TREATMENT	Average number of bacteria
Essential Oils	Before treatment	83.7 \pm 9.2
	Thyme 50%	29.2 \pm 10.1 *
	Eucalyptus 50%	46.1 \pm 17.8
	Oregano 50%	39.5 \pm 5 *
	NSW	66.7 \pm 30.7
	DMSO	284.9 \pm 15.4
Iodine	Before treatment	70.6 \pm 11.1
	3/4 dilution	dead
	1/10 dilution	35.7 \pm 7.5
	1/20 dilution	23.9 \pm 10.5 *
	1/50 dilution	11.7 \pm 5.8 *
	NSW	60.6 \pm 6
Antibiotics	Before treatment	61.3 \pm 30.3
	After treatment	3.8 \pm 3.2 *
	NSW	36.5 \pm 3.2

Table 4 - Inhibition zone diameter of the different treatments. (\pm standard deviation, n=3). *: Sensitive diameters. NSW: natural sea water.

	TREATMENT	Inhibition Diameter (mm)
Control	NSW	No inhibition
	DMSO	No inhibition
Essential Oils	Thyme 50%	41.3 \pm 4.6 *
	Eucalyptus 50%	18 \pm 6.1
	Oregano 50%	44.7 \pm 9.2 *
Iodine	3/4 dilution	22.5 \pm 4.7 *
	1/10 dilution	14.8 \pm 3.1
	1/20 dilution	12.3 \pm 3.1
	1/50 dilution	No inhibition
Antibiotics		No inhibition

Table 5 Taxonomic affiliations of the OTUs impacted by the iodine treatment, compared to their occurrence in the entire iodine dataset.
 * indicates significant p-values after Benjamini-Hochberg correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Taxa	OTUs decreased by iodine treatment			OTUs increased by iodine treatment			Entire dataset	
	Number of impacted OTUs	ratio	p-value	Number of impacted OTUs	ratio	p-value	Number of OTUs	ratio
<i>Acidimicrobiia_unclassified</i>	3	0.04348	0.00003 ***	0	0.00000	0.36363	13	0,00247
<i>Microtrichales_unclassified</i>	2	0.02899	0.00001 ***	0	0.00000	0.09896	3	0.00057
<i>Actinobacteria_unclassified</i>	5	0.07246	0.00001 ***	0	0.00000	0.69409	34	0.00645
<i>Bacteria_unclassified</i>	3	0.04348	0.98989	17	0.09290	0.95748	728	0.13814
<i>Bacteroidetes_unclassified</i>	0	0.00000	0.18926	1	0.00546	0.10729	16	0.00304
<i>Bacteroidia_unclassified</i>	11	0.15942	0.00813 *	27	0.14754	0.00070 ***	421	0.07989
<i>Saprospiraceae</i>	6	0.08696	<0.00001 ***	0	0.00000	0.81904	49	0.00930
<i>Cyclobacteriaceae</i>	0	0.00000	0.50868	20	0.10929	<0.00001 ***	54	0.01025
<i>Cytophagales_unclassified</i>	0	0.00000	0.18926	2	0.01093	0.01876	16	0.00304
<i>Flavobacteriaceae</i>	5	0.07246	0.13318	4	0.02186	0.95541	265	0.05028
<i>Flavobacteriales_unclassified</i>	0	0.00000	0.62309	1	0.00546	0.72888	74	0.01404
<i>Oxyphotobacteria_unclassified</i>	1	0.01449	0.09288	3	0.01639	0.04805	39	0.00740
<i>Pirellulaceae</i>	0	0.00000	0.73690	1	0.00546	0.86741	101	0.01917
<i>Alphaproteobacteria_unclassified</i>	10	0.14493	0.97775	30	0.16393	0.99743	1334	0.25313
<i>Hyphomonadaceae</i>	0	0.00000	0.58088	14	0.07650	<0.00001 ***	66	0.01252
<i>Rhizobiaceae</i>	3	0.04348	0.71437	11	0.06011	0.62325	366	0.06945
<i>Rhodobacteraceae</i>	13	0.18841	0.00039 ***	4	0.02186	0.99767	383	0.07268
<i>Sphingomonadaceae</i>	7	0.08696	0.08099	24	0.13661	0.00041 ***	345	0.06546
<i>Alteromonadaceae</i>	0	0.00000	0.35172	9	0.04918	<0.00001 ***	33	0.00626
<i>Marinobacteraceae</i>	0	0.00000	0.36020	1	0.00546	0.33058	34	0.00645
<i>Haliaceae</i>	0	0.00000	0.68711	11	0.06011	0.00007 ***	88	0.01670
<i>Pseudohongiellaceae</i>	0	0.00000	0.01301 *	1	0.00546	0.00059 ***	2	0.00038
<i>Proteobacteria_unclassified</i>	0	0.00000	0.86364	2	0.01093	0.89527	150	0.02846
TOTAL	69			183			5270	

Fig. 1 Distribution of bacterial OTUs per phylum in the different samples and experiments

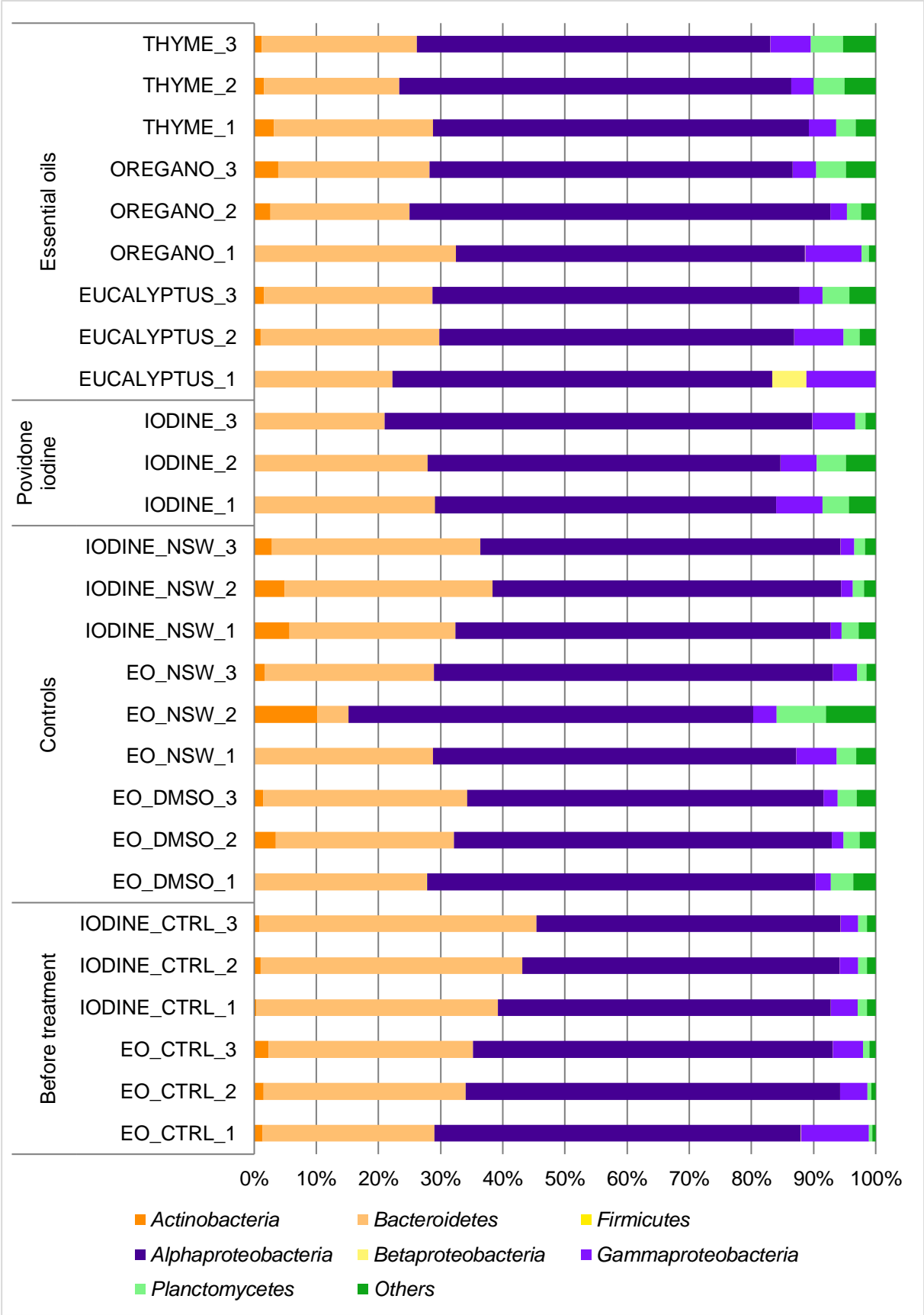
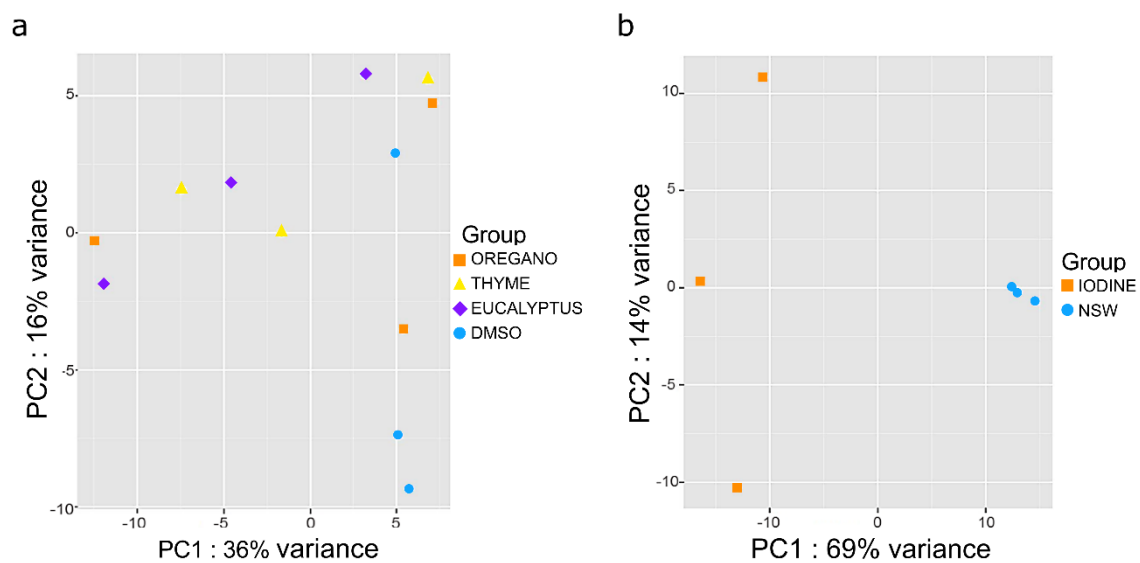


Fig. 2 PCA plot of microbiome composition two weeks after the different treatments (a) Essential oils treatment (b) Povidone-iodine treatment. NSW: Natural Sea Water



SUPPLEMENTARY MATERIAL

Table S1. List of the 252 OTUs significantly different in relative abundance between the treated and non-treated samples (in green are the OTUs that decreased and in red are the OTUs that increased in treated samples). Numbers in parentheses correspond to the confidence of taxonomic affiliations as reported by RDP classifier.

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