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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS & METHODS

Algal cultures

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

Essential oil treatments

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

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

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

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

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

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

Povidone-iodine treatments

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

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

Antibiotic treatments

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

Determination of inhibition diameters

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

Impact of treatments on microbial community

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

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

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

Statistical tests

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

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

RESULTS

Essential oil composition

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

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

Antimicrobial effects of EOs and povidone-iodine in cultures

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

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

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

Effect of treatments on bacterial community composition

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

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

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

DISCUSSION

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

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

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

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

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

Povidone-iodine treatments induce microbial community shifts

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

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

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

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

CONCLUSION AND OUTLOOK

While antibiotic treatments are currently the most efficient way of eliminating algal-associated microbiota and cannot be replaced by any of the tested alternative treatments in the near future, both EOs and povidone-iodine offer promising 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.

DECLARATIONS

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Authors' contributions: Designed study: BBD, SD; performed experiments: BBD; analysed data: BBD, SD; Wrote the manuscript: BBD, SD; Provided valuable input and corrected the manuscript: CB.

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609 # FIGURES AND TABLES

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

610

Table 2 - Ratio after treatment / before treatment of the number of bacteria on the algal surface. (± 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 ± 0.28	_	_	_	_	_	_	_
	Olive oil	+++	_	+++	+++	_	_	_	_
	Eucalyptus	0.38 ± 0.15 *	_	0.30 ± 0.12 *	_	_	3.41	_	_
	Oregano	0.49 ± 0.10 *	_	0.35 ± 0.10 *	_	_	0.79 ± 0.24	_	_
	Thyme	1.10 ± 0.17	_	0.35 ± 0.12 *	_	_	0.44 ± 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 ± 0.22
	ATB	0.06 ± 0.05 *	_	_	_	_	_	_	_
Iodine	NSW	_	_	_	_	_	_	_	0.60 ± 0.22
	30sec	dead	0.55 ± 0.06	_	_	_	0.33 ± 0.01 *	0.62 ± 0.15	0.54 ± 0.14
	1min	dead	0.46 ± 0.02 *	_	_	_	0.43 ± 0.11 *	0.87 ± 0.02	1.35 ± 0.21
	2min	dead	0.89 ± 0.09	_	_	_	0.29 ± 0.16 *	1.01 ± 0.03	17.27 ± 0.12
	10min	_	_	_	0.51 ± 0.11	0.34 ± 0.15 *	0.17 ± 0.08 *	_	0.86 ± 0.08

Table 3 – Average number of bacteria after the treatments and 2 weeks of recovery. (± 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±9.2
	Thyme 50%	29.2±10.1 *
	Eucalyptus 50%	46.1±17.8
	Oregano 50%	39.5±5 *
	NSW	66.7±30.7
	DMSO	284.9±15.4
Iodine	Before treatment	70.6±11.1
	3/4 dilution	dead
	1/10 dilution	35.7±7.5
	1/20 dilution	23.9±10.5 *
	1/50 dilution	11.7±5.8 *
	NSW	60.6±6
Antibiotics	Before treatment	61.3±30.3
	After treatment	3.8±3.2 *
	NSW	36.5±3.2

Table 4 - Inhibition zone diameter of the different treatments. (± 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±4.6 *
	Eucalyptus 50%	18±6.1
	Oregano 50%	44.7±9.2 *
Iodine	3/4 dilution	22.5±4.7 *
	1/10 dilution	14.8±3.1
	1/20 dilution	12.3±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
Acidimicrobiia_unclassified	3	0.04348	0.00003 ***	0	0.00000	0.36363	13	0,00247
Microtrichales_unclassified	2	0.02899	0.00001 ***	0	0.00000	0.09896	3	0.00057
Actinobacteria_unclassified	5	0.07246	0.00001 ***	0	0.00000	0.69409	34	0.00645
Bacteria_unclassified	3	0.04348	0.98989	17	0.09290	0.95748	728	0.13814
Bacteroidetes_unclassified	0	0.00000	0.18926	1	0.00546	0.10729	16	0.00304
Bacteroidia_unclassified	11	0.15942	0.00813 *	27	0.14754	0.00070 ***	421	0.07989
Saprospiraceae	6	0.08696	<0.00001 ***	0	0.00000	0.81904	49	0.00930
Cyclobacteriaceae	0	0.00000	0.50868	20	0.10929	<0.00001 ***	54	0.01025
Cytophagales_unclassified	0	0.00000	0.18926	2	0.01093	0.01876	16	0.00304
Flavobacteriaceae	5	0.07246	0.13318	4	0.02186	0.95541	265	0.05028
Flavobacteriales_unclassified	0	0.00000	0.62309	1	0.00546	0.72888	74	0.01404
Oxyphotobacteria_unclassified	1	0.01449	0.09288	3	0.01639	0.04805	39	0.00740
Pirellulaceae	0	0.00000	0.73690	1	0.00546	0.86741	101	0.01917
Alphaproteobacteria_unclassified	10	0.14493	0.97775	30	0.16393	0.99743	1334	0.25313
Hyphomonadaceae	0	0.00000	0.58088	14	0.07650	<0.00001 ***	66	0.01252
Rhizobiaceae	3	0.04348	0.71437	11	0.06011	0.62325	366	0.06945
Rhodobacteraceae	13	0.18841	0.00039 ***	4	0.02186	0.99767	383	0.07268
Sphingomonadaceae	7	0.08696	0.08099	24	0.13661	0.00041 ***	345	0.06546
Alteromonadaceae	0	0.00000	0.35172	9	0.04918	<0.00001 ***	33	0.00626
Marinobacteraceae	0	0.00000	0.36020	1	0.00546	0.33058	34	0.00645
Halieaceae	0	0.00000	0.68711	11	0.06011	0.00007 ***	88	0.01670
Pseudohongiellaceae	0	0.00000	0.01301 *	1	0.00546	0.00059 ***	2	0.00038
Proteobacteria_unclassified	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

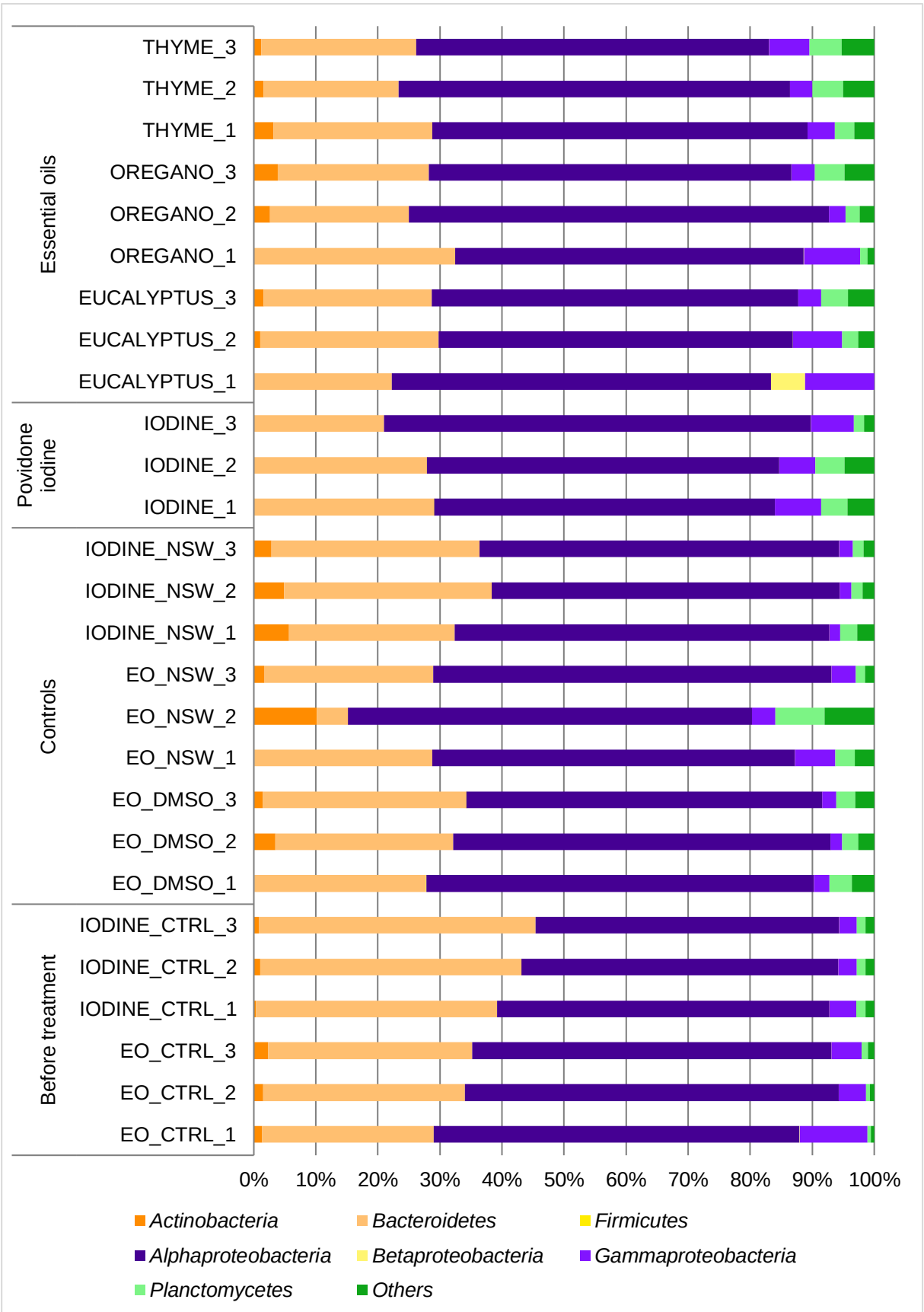
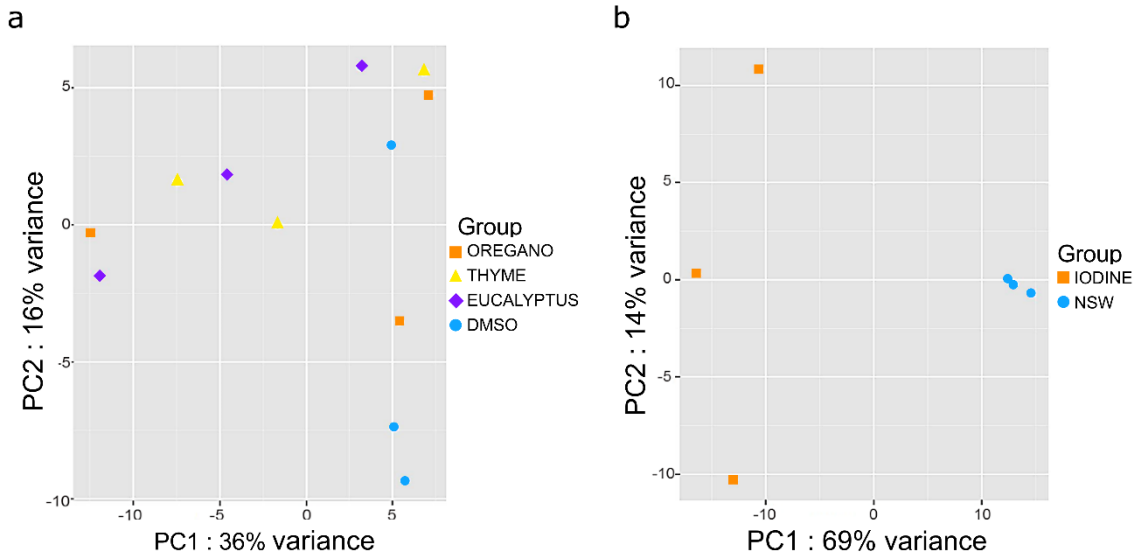


Fig. 2 PCA plot of microbiome composition two weeks after the different treatments (a) Essential oils treatment (b) Povidone-iodine treatment. NSW: Natural Sea Water



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.