# Microbial 'gardening' by a seaweed holobiont: surface metabolites attract protective and deter pathogenic epibacterial settlement

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**Running Head:** Chemical 'gardening' of beneficial epibacteria by an invasive seaweed

**Summary**

1. Epimicrobial communities on seaweed surfaces usually contain not only potentially pathogenic, but also potentially beneficial microorganisms. Capacity of terrestrial plants for chemically mediated recruitment i.e. 'gardening' of bacterial communities in the rhizosphere was recently demonstrated. Empirical evidence directly linking such chemical 'gardening' with the beneficial role of gardened microbes in terrestrial plants is rare and largely missing for aquatic macrophytes.
2. Here we demonstrate that our model invasive seaweed holobiont *Agarophyton vermiculophyllum* possesses beneficial microbiota on its surface that provide

protection from bacterial pathogens. Metabolites from the algal holobiont's surface reduced settlement of opportunistic pathogens but attracted protective epibacterial settlement.

3. We tested 58 different bacterial species (isolated from the surface of *A. vermiculophyllum*) individually in tip bleaching assays. *Kordia algicida* was identified as a 'significant pathogen' inducing a bleaching disease. In addition, 9 other species significantly reduced the risk of algal bleaching and were thus 'significantly protective'. Additionally, 2 'potential pathogens' and 10 'potential protectors' were identified. When 19 significant and potential protectors and 3 significant and potential pathogens were tested together, the protective strains fully prevented bleaching, suggesting that a component of *A. vermiculophyllum's* epimicrobiome provides an associational defence against pathogens. Chemically mediated selective recruitment of microbes was demonstrated in bioassays, where *A. vermiculophyllum* surface metabolites attracted the settlement of protective strains, but reduced settlement of pathogens.
4. Synthesis: The capacity of an aquatic macrophyte to chemically 'garden' protective microorganisms to the benefit of strengthened disease resistance is demonstrated for the first time. Such a role of surface chemistry in 'gardening' of microbes as found in the current study could also be applicable to other host plant – microbe interactions. Our results may open new avenues towards manipulation of the surface microbiome of seaweeds via chemical 'gardening', enhancing sustainable production of healthy seaweeds.

**Key words:** *Agarophyton vermiculophyllum*, Macrophyte, Chemical Defence, Plant-microbe interactions, *Gracilaria vermiculophylla*, Bleaching, Gardening, Invasive, Seaweed, Holobiont.

## Introduction

All eukaryotes including terrestrial plants and aquatic macrophytes are influenced by complex interactions with microbial communities. The animal gut microbiome is very well known to influence the health and nutritional status of its host (Hooper *et al., 2002;* Flint *et al.,* 2012), ultimately forming a metaorganism or holobiont that consists of the host and associated microbiomes (Bordenstein & Theis, 2015). These microbes form an integral part of a plant or animal phenotype, influencing the fitness and ecological traits of their hosts.

The outer body surface is the primary physiological and ecological interface of multicellular aquatic organisms like water plants or seaweeds with the environment (Wahl, 2008). Apart from exchange and uptake of nutrients, this interface is involved in the exchange of chemical cues and signals that mediate the recognition of an organism by a partner, a parasite, an epibiont or a predator. This surface is often colonized by complex microbial communities, a biofilm-like epimicrobiome that has also been denoted as 'second skin' (Wahl *et al.*, 2012). Marine macroalgae i.e. seaweeds have an additional diffusive boundary layer (Hurd, 2000) along with their 'second skin' that serves as the micro-niche of chemically mediated ecological interactions. This micro-niche is analogous to the rhizosphere of plant roots (Hartmann *et al.*, 2009) or the phycosphere of phytoplankton (Bell & Mitchell, 1972).

69 This niche is an ecological interface of seaweed-microbe relationships, modulates most of
70 the interactions between the seaweed host and the environment and is typically
71 characterized by a specific chemical fingerprint.

72 Seaweeds are omnipresent organisms in photic coastal zones, play key roles in carbon
73 fixation, biogeochemical cycling and food web formation. They can drive the biogeochemical
74 pump and release climate cooling gases like dimethyl sulphide (Van Alstyne & Houser,
75 2003). They act as nursery ground and protective shelters for many animals (Schiel & Foster,
76 2006; Pereira *et al.,* 2017). Seaweeds also provide substrate for numerous sessile organisms,
77 ranging from bacteria to macro-invertebrates (Wahl, 1989). Epibacteria that colonize the
78 surfaces of seaweeds vary taxonomically with host, space and time (Cundell et *al.,* 1977;
79 Lachnit *et al.,* 2011) and can affect the well-being of their host in multiple ways.

80 The epimicrobial communities on seaweeds consist not only of pathogenic species but also
81 of potentially beneficial ones. Interactions with the surface epimicrobiome have the
82 potential to influence seaweed health and development in two different ways: they can be
83 detrimental, as seaweeds can be plagued by bacterial and eukaryotic pathogens (see
84 Gachon *et al.,* 2010; Egan *et al.,* 2013 and references therein). The epimicrobiome also often
85 provides inductive settlement cues to algal spores and invertebrate larvae, causing heavy
86 detrimental fouling (see Wahl *et al.,* 2012 and references therein). Alternatively, seaweed
87 epimicrobiomes can also be beneficial, supplying essential nutrients (see Hollants *et al.,*
88 2013 and references therein) and chemical cues for morphogenesis (see Wichard *et al.,*
89 2015 and references therein).

90 A suspected yet relatively undemonstrated beneficial role of the epimicrobiome is the
91 protection from pathogens and other detrimental microorganisms (but see Longford *et al*.,

2019). A certain component of these epimicrobial communities on seaweed surfaces is quite host-specific (Lachnit *et al.,* 2009; Bengtsson & Ovreas, 2010) and the same is true for the rhizosphere of terrestrial plant roots (Raaijmakers *et al.,* 2009). However, the principles governing the assemblages of microbes on surfaces of seaweeds or any other aquatic macrophytes are unclear. Based upon recent independent studies with terrestrial plants and aquatic macrophytes the following models for the association of microbial communities have been proposed: 1. The 'neutral' hypothesis assumes that species are ecologically equivalent, and the community structure is determined randomly (Hubbel 2001, 2006; Woodcock, 2007). 2. The 'niche' model stresses that only microorganisms which are adapted to the specific conditions on a host surface will be able to settle on it (Dumbrell *et al.,* 2010). 3. The 'lottery' hypothesis combines both neutral and functional aspects and predicts that multiple microorganisms could make use of the same niche, but those that reach it first have a larger chance of settlement success (Burke *et al.,* 2011). 4. Untargeted recruitment of microorganisms by the host via the release of exuded nutrients has also been proposed, as well as targeted deterrence by processes like induced defence (Weinberger, 2007).

By comparing bacterial root microbiomes between wildtype *Arabidopsis thaliana* and mutants that could not produce the defence phytohormone salicyclic acid, Lebeis *et al.,* 2015 recently demonstrated that salicylic acid signalling can modulate root microbial communities. While such studies on the role of chemical manipulation of root microbiota have started to appear for land plants, no parallel study exists for aquatic macrophytes that demonstrates an active 'deliberate' recruitment or 'gardening' of beneficial microbes. Surface associated metabolites may shape the microbial communities on seaweed surfaces. For example, halogenated furanones excreted by the host *Delisea pulchra* were

demonstrated to shape the microbiome of the seaweed (Longford *et al.,* 2019). Also, in the brown alga *Fucus vesiculosus* surface metabolites were found to have an effect on the biofilm composition both under field and lab conditions (Lachnit *et al.,* 2010). The authors used an experimental system that simulated the delivery of *Fucus* surface associated metabolites on artificial substrates and tested the effect of algal surface chemistry on bacterial community composition. Bacterial communities that developed on test surfaces loaded with *Fucus* surface metabolites were found to be quite similar to communities on the surfaces of *Fucus*, but different from communities on solvent controls, which hinted at the strong selective force of these surface metabolites of *Fucus*. However, for the investigation with *Fucus vesiculosus* no evidence could be demonstrated for the beneficial role of such microbes and thus the purpose of such chemically mediated recruitment of microbes. Also, studies of the rhizosphere of terrestrial plants already reported selective 'gardening' of microbes (Currier & Strobel, 1976; Bacilio-Jim´enez *et al.,* 2003). For example, root exudates of different developmental stages of *Arabidopsis* promoted the formation of microbial communities with different compositions when the influence of environmental and soil edaphic factors was experimentally excluded (Yuan *et al.,* 2015). Although there have been demonstrations of the possible beneficial roles of such active microbial gardening for plant growth and development in terrestrial environments (Lebeis *et al.,* 2015) and marine environments (Kessler *et al.,* 2018), none of the studies in the aquatic realm have yet been able to empirically link chemically mediated microbial 'gardening' with resistance to disease.

Thallus whitening, bleaching or '*ice-ice* disease' is a common problem in certain farmed and wild red seaweeds, such as *Gracilaria 'conferta'* (Weinberger *et al.,* 1994*;* Weinberger *et al.,*

1997*)*, *Kappaphycus* and *Eucheuma* or *Delisea pulchra* (Case *et al.,* 2011; Campbell *et al.,* 2011). It was repeatedly shown that this depigmentation symptom can be induced by multiple opportunistic bacterial pathogens and in the case of *G. 'conferta'* a component of the microbiome was shown to prevent the disease. Also, in *Delisea pulchra* early successional epibacterial strains protected the host from a later successional strain that was pathogenic when the host microbiome was experimentally disturbed (Longford *et al.,* 2019). In the context of the 'gardening' hypothesis the present study investigated whether (a) epibacteria originating from healthy specimens of the invasive red seaweed *Agarophyton vermiculophyllum* can also induce thallus bleaching in *A. vermiculophyllum*, whether (b) a subset of epibacterial strains of the algal microbiome offers protection towards pathogenic strains and whether (c) *A. vermiculophyllum* has a capacity for chemically mediated recruitment of such protective microbes while deterring the settlement by pathogens.

## Materials and Methods

### Isolation and identification of epibacterial strains

Five invasive and five native populations of *Agarophyton vermiculophyllum* (Gurgel *et al.*, 2018) (Synonym: *Gracilaria vermiculophyllum* (Ohmi) Papenfuss, hereafter: *Agarophyton*) were sampled along the Danish-German Peninsula of Jutland and Schleswig-Holstein and the South Korean peninsula, respectively (see Table S1 in Supporting Information). Using standard protocols, bacterial strains were isolated from the surface of *Agarophyton.* Thus, the tested bacterial strains were ecologically relevant. 5 g of pooled algal individuals arising from each population were rinsed three times in 35 ml of Bacto Marine Broth (MB; Difco 2216, Becton Dickinson and Company, Heidelberg, Germany) to remove loosely attached bacteria. Then, the samples were immediately transferred to 10 ml of MB and vortexed vigorously for 20 s to detach the epibacteria. The suspension was subsequently diluted in

MB using the log dilution method and plated out directly on MB agar (37.3 $g^{-1}$ MB, 15.0 $g^{-1}$ agar; pH 7.6) in standard Petri dishes. Incubation was performed in the dark at 28°C for 7 days. Pure cultures were obtained through several subsequent picking and culturing steps for individual colonies on MB agar plates. The isolates were cryopreserved at -80°C using the Cryobank System (Mast Diagnostica GmbH, Reinfeld, Germany) according to the manufacturer's instructions, until processed further. Strains were identified by 16S rRNA sequencing as described in Saha *et al.,* (2016) and tested in the bioassays described below. For methodological details see Appendix S1 in Supporting Information.

***Agarophyton* tip bleaching assay with epibacterial strains**

**(A) Bleaching assay with single isolates**

To test the potential capacity of epibacterial strains for induction of thallus bleaching in *Agarophyton*, 58 of the cryopreserved bacterial strains were reanimatedin November 2015. They were then maintained on MB agar medium in darkness. All cultures were incubated at 25°C, except *Psychroserpens mesophilus* and *Pseudoalteromonas lipolytica* as they exhibited no growth at this temperature and were incubated at 15°C.

In November 2015, *Agarophyton* individuals were sampled from Nordstrand (53°29'10.25"N, 8°38'35.33"E) and brought to the laboratory in a cooler box. They were maintained in 20 L aquaria at a salinity of 33 psu (approximate salinity value at the collection site) and a temperature of 16$^{o}$ C under constant aeration and a photon flux density of 75 µmol $m^2$ $s^1$ (12 h of light per d).

For the experiment, *Agarophyton* thallus tips (n = 6 in total for each bacterial strain, each tip was ca. 2-3 cm long) were individually placed into separate wells of 24 well plates (Sarstedt, GmbH) containing 2 ml of sterile sea water (SSW, 33 psu). To eliminate epibacteria from the algal surface, two antibiotics, Vancomycin and Cefotaxim (each at concentration of 0.1 mg

187 $ml^{-1}$) were added to each well (Weinberger et al., 1997). The wells were then incubated for 2
188 days at 16 °C and a photon flux density of 75 µmol $m^{-2}$ $s^{-1}$. Following this pre-treatment, the
189 wells were carefully emptied of SSW and antibiotics. Remaining antibiotics were removed
190 from *Agarophyton* tips and the wells by washing with 1 ml of SSW. Finally, 2 ml of SSW were
191 again added into each well and bacteria cultures were immediately inoculated.
192 Prior to inoculation all bacterial cultures were grown in sterile MB medium for 3-7 d at the
193 same temperature that was used for their maintenance (25°C or 15°C, see above) in
194 darkness until they had reached an $OD_{610}$ of 0.2 to 0.3. A volume of 20 µl bacterial cells
195 along with the medium was then added into the wells containing *Agarophyton* tips (n = 6).
196 Controls consisted of the same volume of sterile bacterial culture medium added into the
197 wells containing *Agarophyton* tips (n = 6) and treated in a similar manner as above.
198 Following five days of incubation (16 °C and a photon flux density of 75 µmol $m^{-2}$ $s^{-1}$) all wells
199 were checked under the binocular microscope (magnification factor: 20, see Supporting
200 Information Fig. S1) and numbers of bleached and non-bleached tips in each well were
201 counted, using a black background (Weinberger *et al.,* 1997).
202 Relative risk of thallus tip bleaching in treatments with addition of bacteria relative to
203 control treatments were calculated as odds ratios of numbers of bleached and non-
204 bleached tips.
205
206 $$\text{Relative risk of bleaching} = \frac{(\text{Bleached tips in treatments} \div \text{healthy tips in treatments})}{(\text{Bleached tips in controls} \div \text{healthy tips in controls})}$$
208 95% Confidence intervals of these ratios were constructed following Fisher and Van Belle
209 (1993). The divergence of these ratios from 1 (= no effect of the treatment on the risk) was
210 tested for significance, using Fisher's exact test (Fisher & Van Belle, 1993). Isolates that

211 significantly induced thallus tip bleaching were retested in one or two independent
212 repetitions of the experiment to confirm the result. The Mantel-Haenßel-extention of
213 Fisher's exact test for replicated test designs was used for the statistical analysis in these
214 cases (Fisher & Van Belle, 1993; Weinberger *et al.,* 1997). To reduce the risk of type I error a
215 Bonferroni correction was applied if multiple tests had to be done (Fisher & Van Belle,
216 1993). Isolates that turned out to be significantly pathogenic after applying Bonferroni
217 correction (i.e. $p<0.00086$) were called 'significant pathogens' while the ones which were
218 non-significant after Bonferroni correction (i.e. $p<0.05$) were called 'potential pathogens'.
219 Isolates that reduced the risk of thallus tip bleaching were all designated as 'protectors'.
220 However, isolates that significantly reduced the risk of thallus tip bleaching after applying
221 Bonferroni correction (i.e. $p<0.00086$) were called 'significant protectors', while the other
222 isolates that also reduced tip bleaching ($p<0.05$) but were not significant after applying
223 Bonferroni correction were designated as 'potential protectors'.

224 **(B) Bleaching assay with combined 'protectors' and 'pathogens'**

225 The combined effect of all 'protectors' ('significant' and 'potential' protectors) on the
226 virulence of confirmed pathogens ('significant' and 'potential' pathogens) identified in the
227 above experiment was tested in an additional experiment. In order to observe any
228 community effect of these epibacteria, we included all the significant and potential strains
229 because bonferroni correction is known to be relatively conservative (Moran 2003). The
230 general design was as described above, but the method of inoculation differed: bacterial
231 cultures were incubated until their $OD_{610}$ was between 0.1 and 0.5. Different aliquots of all
232 identified 'protectors' were then pooled so that each culture contributed the same $OD_{610}$ to
233 the mixture, which had a final $OD_{610}$ of 0.25. Cells in the mixture were separated from the
234 medium by centrifugation (10 000 g, 20 min) and resuspended in SSW. A mixture of three

pathogens was prepared in an analogous manner. *Agarophyton* was then inoculated with 10 µl of either (i) all of the 'protectors' (19 bacterial strains, thereof 10 'significant protectors' and 9 'potential protectors') or (ii) all of the pathogens (3 bacterial strains, thereof 1 'significant pathogen' and 2 'potential pathogens') or (iii) pathogens and protectors together (22 bacterial strains). Final volumes of either protectors or pathogens were brought up to 20 µl with SSW, while controls received just 20 µl of SSW. This experiment was repeated in one fully independent repetition (n = 2 x 6). Numbers of bleached thallus tips relative to all tips were counted and significant differences were identified using Kruskal-Wallis-ANOVA and Dunn's post hoc test.

**Extraction of surface associated metabolites of *Agarophyton***

To generate surface associated metabolites, *Agarophyton* individuals (n=5) were collected from the same location as above, i.e. Nordstrand. Surface-associated metabolites originating from single *Agarophyton* specimens were extracted immediately upon collection according to Saha *et al.,* (2016). Briefly, *Agarophyton* branches were dipped into a solvent mixture of dichloromethane and hexane 1:4 (v/v) for 5 s. This process is benign and does not harvest intracellular metabolites (see Saha *et al.,* (2016) for details). The prepared extract (n=5) containing the surface associated metabolites was filtered through GF/A filter paper (Whatman Ø = 15 mm) to remove particles, and the solvent was evaporated under a vacuum at 20°C, using a rotary evaporator (Laborota 3000, Heidolph, Germany). The extract was then taken up in acetonitrile in such a way that 1.5 µl contained metabolites extracted from an algal surface of 99.64 mm². The extract was used to coat each replicate well with a surface area 99.64 mm². Acetonitrile was then evaporated and metabolites originating from the surface of the alga remained on the surface of the well, allowing us to test at an ecologically realistic 1-fold concentration.

**Defence capacity test of *Agarophyton* surface metabolites against pathogens and protectors**

Inhibition or reduction of bacterial settlement and attachment represents the first line of defence against microbial challenge (Lane & Kubaneck, 2009). Thus, an antisettlement assay was employed as the most relevant criterion for determining antimicrofouling defence because it quantifies both repellent and toxic effects (Wahl, Jensen & Fenical, 1993). The assay was performed according to Saha *et al.* (2016). Briefly, the assay was conducted in 96-well plates that were surface-impregnated with *Agarophyton* surface extract metabolites at a 1-fold natural concentration (Saha *et al.,* 2016) and with solvent residue as a control. In total five extracts – originating from five *Agarophyton* individuals - were tested and regarded as replicates. Each *Agarophyton* extract was then subdivided and tested in three pseudo replicates against each bacterial isolate to account for the variability in the bacterial settlement rates. Results obtained for pseudo replicates were averaged before statistical analyses were conducted. The tested target strains were chosen based on results from the tip bleaching assay described above. All three pathogens (both the 'significant pathogen' *Kordia algicida* and the 'potential pathogens' *Croceitalea eckloniae, Pseudoalteromonas arctica*) were tested in the anti-settlement assays, but due to shortage of surface extracts was it not possible to test all nineteen 'protective' strains. Thus, only five 'significant protectors' i.e. *Ralstonia sp., Shewanella aquimarina, Tenacibaculum skagerrakense, Alteromonas stellipolaris, Tenacibaculum aestuarii* and two 'potential protectors' i.e. *Cobetia marina* and *Nonlabens dokdonensis* were tested. 106 µL suspensions of these bacterial strains (O.D. 0.6-0.8) precultured in MB liquid medium (as described above) were pipetted into the wells. The bacteria were allowed to settle for 3 h, and the settled cells that

could not be removed by rinsing two times with 110µL sterile seawater were stained with the fluorescent DNA-binding dye Syto 9 (Invitrogen, GmbH). Fluorescence was subsequently measured (excitation, 477–491 nm; emission, 540 nm) with a plate reader as a proxy for bacterial settlement in terms of the attached cell density. All tested strains were allowed to settle on all extracts.

The defence strength of *Agarophyton* surface metabolites is expressed as the 'log effect ratio,' i.e., the logarithm of the fluorescence attributable to the settled bacteria of strain Y in the presence of surface metabolites, divided by the fluorescence attributable to the settled bacteria of strain Y in the absence of surface metabolites. A log effect ratio value of 0 (i.e., an equal number of settled bacteria in wells with and without surface metabolites) indicated that the tested surface metabolites had no effect on settlement, whereas a negative log effect ratio value indicated a deterrent effect, and a positive log effect ratio value indicated an attractant effect. Thus, a log effect ratio of -1 represents a 10-fold reduction, whereas a value of +1 represents a 10-fold enhancement of bacterial settlement caused by surface metabolites.

$$\text{Defence strength} = \log\left(\frac{\text{bacterial settlement in presence of } \textit{Agarophyton} \text{ surface metabolites}}{\text{bacterial settlement in absence of } \textit{Agarophyton} \text{ surface metabolites}}\right)$$

## Results

### *Agarophyton* tip bleaching assay with epibacterial strains

#### (A) Bleaching assay with single isolates

Out of 58 tested bacterial isolates *Kordia algicida* was found to significantly increase the risk of tip bleaching (Table 1; Fig. 1, $p<0.00086$), compared to control treatments without inoculation of bacteria and was a 'significant pathogen' after Bonferroni correction. Two additional isolates (*Pseudoalteromonas arctica* and *Croceitalea eckloniae*) had the same effect but were not significantly pathogenic after Bonferroni correction (Table 1; Fig. 1, $p<0.05$) and were thus 'potential pathogens'. Out of the remaining 55 isolates, 9 were found to significantly reduce the risk of tip bleaching (Table 1; Fig. 1, $p<0.00086$) and were grouped under 'significant protectors'. 10 others had the same effect, although they were not significantly protective after Bonferroni correction (Table 1; Fig. 1, $p<0.05$) and were called 'potential protectors'. The remaining 36 isolates were found to be neutral, neither inducing nor preventing bleaching (see Table S2; Fig. S2 in Supporting Information).

Similar numbers of microbiota that originated from native and non-native populations (30 and 28, respectively, see Table 1 and Table S2) of *Agarophyton* were tested in our bleaching assay and double numbers of protective microbiota were detected from the non-native range (3 from the native range and 6 from the non-native range, respectively, Table 1).

**(B) Bleaching assay with combined 'protectors' and 'pathogens'**

When all three isolates (one 'significant pathogen' and two 'potential pathogen') that induced bleaching individually at least with $p < 0.05$ were combined, a significant increase in bleaching relative to the control was again observed (Fig. 2, $p<0.05$). However, combined application of these three 'pathogens' and the nineteen 'protective' isolates that prevented bleaching individually at least with $p < 0.05$ resulted in no such increase (Fig. 2, $p<0.05$). No bleaching was observed when all 19 'protectors' and no 'pathogens' were inoculated.

**Defence capacity test of *Agarophyton* against pathogens and protectors**

329 The effect of *Agarophyton* surface associated metabolites on bacterial settlement differed
330 significantly between the two groups of bacteria, i.e. 'protectors' and 'inducers' (Fig. 3,
331 Welch-corrected t-test, $p < 0.0001$). While the surface associated metabolites significantly
332 increased the settlement of 'protectors', the settlement of the bleaching 'inducers' was
333 significantly reduced by the surface associated metabolites.

334

335 **Discussion**

336 The data presented here demonstrate for the first time that aquatic macrophytes can use
337 surface associated chemicals not only to directly reduce settlement of pathogenic bacteria,
338 but also to recruit bacterial strains that provide protection from such pathogens. The
339 epimicrobiome of *Agarophyton* contains a component that protects the alga from
340 pathogens in a similar way as earlier demonstrated for two other seaweeds (*G. conferta*
341 (Weinberger *et al.,* 1997) and *D. pulchra* (Longford *et al*., 2019)), and the settlement of such
342 protective bacteria on the surface of *Agarophyton* is not random. Surface associated
343 metabolites from the *Agarophyton* holobiont significantly deterred three strains that were
344 significantly and potentially pathogenic, while the metabolites had a probiotic effect
345 towards seven significantly and potentially protective strains that were tested. This confirms
346 the surface chemistry of *Agarophyton* has a similarly strong selective effect on bacterial
347 colonization as in *Fucus vesiculosus* (Lachnit *et al.,* 2010) or *Delisea pulchra* (Longford *et al.,*
348 2019). Moreover, it demonstrates for the first time that this selection is not only targeted to
349 exclude pathogens, but also targeted to attract protectors. Together with Lachnit *et al.,*
350 (2010) and Kessler *et al.,* (2018) our data strongly support the concept of chemically
351 mediated recruitment of microbes and not the 'neutral' hypothesis, according to which the
352 microbial community structure is determined randomly. Our data clearly support the

353 targeted deterrence hypothesis, as settlement of detrimental bacteria was chemically
354 suppressed. On the other hand, we cannot reject the 'niche' model, as multiple microbiota
355 were attracted by *Agarophyton* and possibly able to make use of resources provided by it.
356 Also, the 'lottery' hypothesis cannot be currently rejected, since the capacity of attracted
357 microbiota to coexist and share host resources is unknown.

358 Only 5% of the marine bacterial strains are cultivable (Haglund et al., 2002) and to date no
359 alternative technique has been developed to separate selected microbial components from
360 natural microbial communities and to test them in infection assays. Thus, only a small
361 fraction of all bacteria that are associated with the surface of *Agarophyton* could be isolated
362 and tested in our study. One representative out of 58 tested bacterial species, *Kordia*
363 *algicida*, was significantly capable to induce the tip bleaching symptom in *Agarophyton*. *K.*
364 *algicida* is already known to be detrimental to other organisms. It can kill diatom blooms in
365 a protease mediated molecular interaction (Paul & Pohnert, 2011) and a similar mechanism
366 cannot be excluded in the present case. Bleaching is often correlated with microbial cell wall
367 matrix degrading activity (Weinberger *et al.,* 1994, 1997), but this was not the case in the
368 present study, as *Kordia* is incapable of agar degradation.

369 Two other isolates – which were also not agar degraders - also exhibited the potential to
370 induce bleaching symptoms in *Agarophton*, which strongly suggests that this capacity is not
371 unique. Interestingly, all three detrimental isolates originated from virtually healthy host
372 specimens. Thus, a relevant fraction of *Agarophyton*'s surface microbiome is obviously
373 composed of opportunistic pathogens that can induce bleaching symptoms under certain
374 conditions, similar as in several red seaweeds belonging to other species (Case *et al.,* 2011;
375 Weinberger *et al.,* 1994; Weinberger *et al.,* 1997). Given that three out of 58 culturable

strains were significant or potential pathogens this fraction can be estimated to include approximately 5 % of the microbiome. However, this percentage calculation is based on the culturable proportion which is just a representative sample of the whole microbiome.

Of the remaining strains, 19 (9 ‘significant protectors’ and 10 ‘potential protectors’) could reduce the risk of ‘spontaneous’ bleaching in thalli that were not intentionally inoculated with pathogenic bacteria (Fig. 1). All the specimens of *Agarophyton* tested in our bleaching induction assays were subjected to a pretreatment with two antibiotics that inhibited bacterial cell wall synthesis, with the dual goal to remove opportunistic pathogens and to disturb and weaken any protective components of the algal microbiome. The circumstance that bleaching occurred ‘spontaneously’ at a low rate but could be prevented by an important percentage of all tested isolates suggests that some opportunistic pathogens survived the treatment with antibiotics but could then not become virulent when protective bacteria were inoculated - similar as previously reported for bacteria that had been isolated from *Gracilaria ‘conferta’* and prevented thallus tip bleaching in this alga (Weinberger *et al.,* 1997). The protective effect of various isolates on *Agarophyton* was further confirmed when all the 19 protective strains (nine ‘significantly protective’ and ten ‘potentially protective’) were tested together in combination with the 3 pathogenic strains (one ‘significant pathogen’ and two ‘potential pathogens’) and a bleaching reduction was still documented. Alltogether, our observations strongly hint at the presence of protective epibacteria on the surface of *Agarophyton.* They could (again estimated from the number of identified isolates in our tested strain collection) comprise at least 15% of all taxa present in this microbiome. The presence of such beneficial bacteria has been previously demonstrated not only for other Gracilarioids (Weinberger *et al.,* 1997), but also for *Delisea pulchra* (Longford et al., 2019), corals (Rosenberg *et al.*, 2007) and other seaweeds like the brown alga *Fucus*

400 *vesiculosus,* in which surface associated bacteria were found to inhibit the settlement of
401 macrofoulers (Nasrolahi *et al.,* 2012).

402 The biofilms on seaweed surfaces represent highly competitive environments, with
403 microbes competing for refuge, nutrients and substratum, and interspecific antagonistic
404 effects of bacterial strains are not rare. For example, such effects have been previously
405 demonstrated for the brown alga *Saccharina latissima* (Wiese *et al.,* 2009), the red alga
406 *Delisea pulchra* and the green alga *Ulva australis* (Penesyan *et al.,* 2009). Release of
407 inhibitory components like antibiotics (Wiese et al., 2009) and/or quorum sensing inhibitors
408 (Romero et al., 2010) has been observed and could also explain the 'protective' effect
409 observed by us. Interestingly, one of the significant protective strains, *Pseudoalteromonas*
410 *piscicida*, belongs to a genus which comprises several species that are known to produce
411 antibacterial products to outcompete other bacteria for space and nutrients (Holmström &
412 Kjelleberg, 1999). Also *Pseudoalteromonas piscicida* has been recently demonstrated to
413 inhibit and/or kill competing bacteria - including several marine pathogens, such as *Vibrio*
414 *vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Photobacterium damselae*, and
415 *Shewanella algae* - through secretion of antimicrobial substances and the direct transfer of
416 digestive vesicles to competing bacteria (Richards *et al.,* 2017). On the other hand,
417 *Shewanella aquimarina* exhibited a strong protective effect against *Agarophyton* tip
418 bleaching in the current study and the same was observed with two other potentially
419 protective species of the genus *Shewanella, i.e. S. marisflavi* and *S. loihica.* These
420 observations contrast with the findings that *S. marisflavi* is a pathogen of sea cucumbers (Li
421 *et al.,* 2010) and other bacteria of the genus are pathogenic towards humans. The
422 mechanisms behind the protective effects on *Agarophyton* deserve further investigation.
423 The exact (additive or synergistic) contributions of the active epibacterial players in the

424 cross-infection experiment with all 19 ‘protectors’ combined with 3 ‘pathogens’ are not
425 known yet.

426 Beneficial roles of certain components in the bacterial communities around the rhizosphere
427 of terrestrial plants are well known. They can not only facilitate nutrient acquisition, but also
428 support plant growth under biotic and abiotic plant stress (Lareen *et al.,* 2016*;* Mendes *et*
429 *al.,* 2013). Seaweed-associated bacteria may also facilitate nutrient acquisition and provide
430 essential vitamins and growth factors (Wahl *et al.*, 2012), and – as confirmed in the present
431 study – mediate biotic stress. However, while we have started to understand these benefits
432 and to gather evidence of a selective recruitment of bacteria both in terrestrial (Lebeis *et al.,*
433 2015) and aquatic environments, empirical links between this selective recruitment of
434 communities and a health benefit for the host are still very rare in the aquatic realm. Kessler
435 *et al.,* 2018 recently demonstrated for the marine macroalga *Ulva mutabilis* (Chlorophyta) a
436 mediating role of the algal osmolyte DMSP (dimethylsulfoniopropionate) in the attraction of
437 the beneficial bacterium *Roseovarius* sp. MS2, responsible for release of morphogenetic
438 compounds that ensure proper algal morphogenesis. In absence of these morphogenetic
439 compounds under axenic conditions, *Ulva mutabilis* develops into callus-like colonies
440 consisting of undifferentiated cells and abnormal cell walls. While microbial ‘gardening’ via
441 use of chemicals has been documented in terms of growth and development of seaweeds
442 (Kessler *et al.,* 2018), our study demonstrates for the first time such a link between the
443 disease resistance capacity of a seaweed and beneficial selective gardening of ‘protective’
444 bacteria based upon surface chemistry.

445 Metabolites present on the surface of seaweeds or in the rhizosphere are a cocktail of
446 metabolites originating both from the algal or plant host and from associated surface

447 microbiota. Such surface associated metabolites from the algal holobiont are also known to
448 function as a defence against fouling by microfoulers (e.g. bacteria, diatoms) and
449 macrofoulers (e.g. barnacle larvae, mussels) (reviewed by Da Gama, 2014; Saha *et al.,* 2017).
450 Also epibacteria from seaweeds are well known to have inhibitory activities against other
451 fouling organisms (Singh & Reddy, 2014). Thus, it is possible that beneficial bacteria
452 recruited by *Agarophyton* will not only act as a defence against pathogens but also against
453 other foulers, like filamentous algae. Using a transcriptomic approach, de Oliviera et al.,
454 2012 demonstrated that the red seaweed host *Laurencia dendriodea* (rather the surface
455 associated bacteria) is involved in the biosynthesis of terpenoids (chemical defence
456 compounds against bacterial colonization and infection) through the mevalonate
457 independent pathway. For the *Agarophyton* holobiont, we do not know yet the identity of
458 surface associated bioactive compounds. Thus, it was not possible for us to distinguish the
459 relative contributions of surface metabolites originating from the algal host *Agarophyton*
460 and from surface associated microbiota. Mutants of *Arabidopsis thaliana* with suppressed
461 salicylic acid signalling pathways formed abnormal root microbiomes when compared to the
462 wild plants (Lebeis *et al.,* 2015), which could suggest that the role of the host was more
463 important in this specific case. The contribution of seaweed microbiome metabolites
464 depends on the community composition, abundance and metabolic activity (Wahl *et al.,*
465 2010) and may be expected to be more variable than that of the host. Selective effects
466 observed with surface associated metabolites coming from different algal individuals varied
467 relatively little in our study, which could suggest that metabolites generated by the host
468 have more importance. However, our knowledge of the species-species interactions of
469 cultivatable and non-cultivatible taxa associated with *Agarophyton* or other plants is
470 rudimentary at best. The involvement of multiple protective microorganisms in our and

several other cases (see above) strongly supports the view that the traditional conceptual model emphasizing direct interactions of hosts and single microbes needs to be expanded to a holobiont concept if seaweed- or plant-microbe interactions are to be understood (Egan *et al.*, 2013).

In conclusion, our study demonstrates for the first-time selective chemical 'gardening' of protective epibacterial strains by a seaweed holobiont with regard to disease resistance capacity. The combined effect of metabolites generated by the host alga and the protection offered by associated microbial partners determines the virulence of harmful opportunistic bacterial pathogens. A major component of the epibacterial community appears capable of contributing to this protection against co-occurring pathogens, which suggests that microbiota of very different taxonomic groups may provide the holobiont with the same ecological function, which could be pivotal for the establishment of *Agarophyton* in new environments. Thus, absence of protective microbiota in new environments might not be a factor limiting the invasion success of *Agarophyton*.

As known for other seaweeds like the brown alga *Fucus vesiculosus*, bioactive surface metabolites often act in synergism or additively and/or antagonistically, producing an overall defensive or prebiotic effect on bacterial recruitment (Saha *et al.*, 2011; Saha *et al.,* 2012). The identification of metabolites responsible for such chemical 'gardening' effects via classical bioassay guided fractionation techniques in the near future may allow us to manipulate algal thallus microbiomes to enhance seaweed health, prevent bleaching diseases and ensure production and sustainability in *Agarophyton* aquaculture.

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497 **Author contributions**

498 M.S. isolated and identified the bacterial isolates, designed and performed the anti-
499 settlement experiments. F.W. designed and performed the tip bleaching assays. M.S. and
500 F.W. analysed the data. M.S. wrote the paper and F.W. contributed to the editing.

501 **Author Declaration**

502 The authors declare no conflict of interest. Data underlying this publication are freely
503 accessible and can be downloaded from the DRYAD data repository (Provisional DOI:
504 doi:10.5061/dryad.52j8p1r).

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644

645

646 **Tables**

647 Table 1. Epibacterial strains [isolated from *Agarophyton vermiculophyllum* (GV) populations]
648 that were tested in tip bleaching assays. IR=bacteria isolated from the invasive populations
649 of GV; NR=bacteria isolated from the native populations of GV. Significantly protective
650 strains are bacteria that were still protective after Bonferroni correction. Asterisks indicate
651 strains that were tested in anti-settlement assays with surface associated compounds of GV.

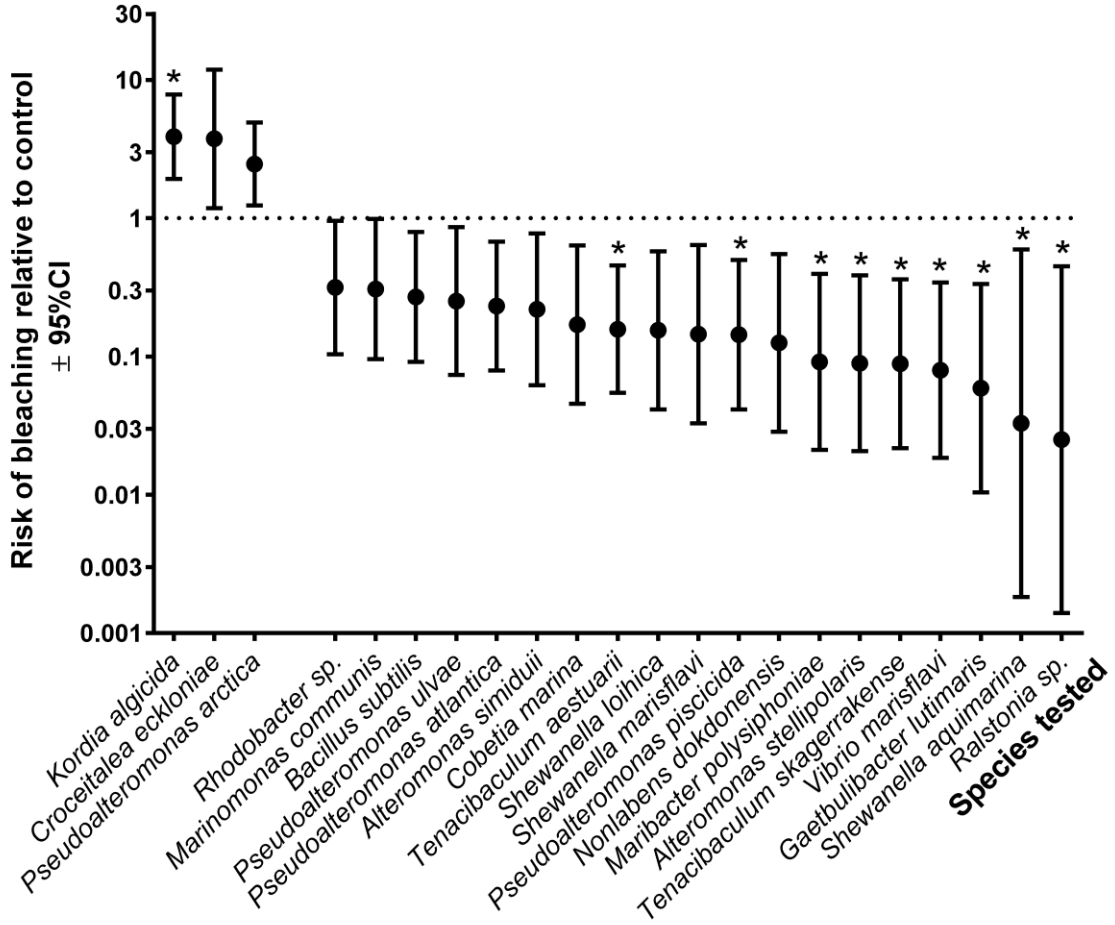
| **Isolate code, Range of isolation** | **Closest match on RDF** | **Designation** |
|---|---|---|
| G-NY6, IR | *Ralstonia sp.** | Significantly Protective |
| G-JI1, NR | *Shewanella aquimarina** | Significantly Protective |
| G-NORD3, IR | *Gaetbulibacter lutimaris* | Significantly Protective |
| G-G2, NR | *Vibrio marisflavi* | Significantly Protective |
| G-MAN7, IR | *Tenacibaculum skagerrakense** | Significantly Protective |
| G-HO9, IR | *Alteromonas stellipolaris** | Significantly Protective |
| G-ODO3, NR | *Maribacter polysiphoniae* | Significantly Protective |
| G-FALK1, IR | *Nonlabens dokdonensis** | Potentially Protective |
| G-NORD11, IR | *Pseudoalteromonas piscicida* | Significantly Protective |
| G-G4, NR | *Shewanella marisflavi* | Potentially Protective |
| G-DA3, NR | *Shewanella loihica* | Potentially Protective |
| G-NORD6, IR | *Tenacibaculum aestuarii** | Significantly Protective |
| G-JI4, NR | *Cobetia marina** | Potentially Protective |
| G-NY1, IR | *Alteromonas simiduii* | Potentially Protective |

| | | |
|---|---|---|
| G-DA5, NR | *Pseudoalteromonas atlantica* | Potentially Protective |
| G-FALK2, IR | *Pseudoalteromonas ulvae* | Potentially Protective |
| G-MAN5, IR | *Bacillus amyloliquefaciens* | Potentially Protective |
| G-JI5, NR | *Marinomonas communis* | Potentially Protective |
| G-HO8, IR | *Rhodobacter sp.* | Potentially Protective |
| G-MAN6, IR | *Pseudoalteromonas arctica** | Potentially Pathogenic |
| G-NORD9, IR | *Croceitalea eckloniae** | Potentially Pathogenic |
| G-MAN4, IR | *Kordia algicida** | Significantly Pathogenic |

652

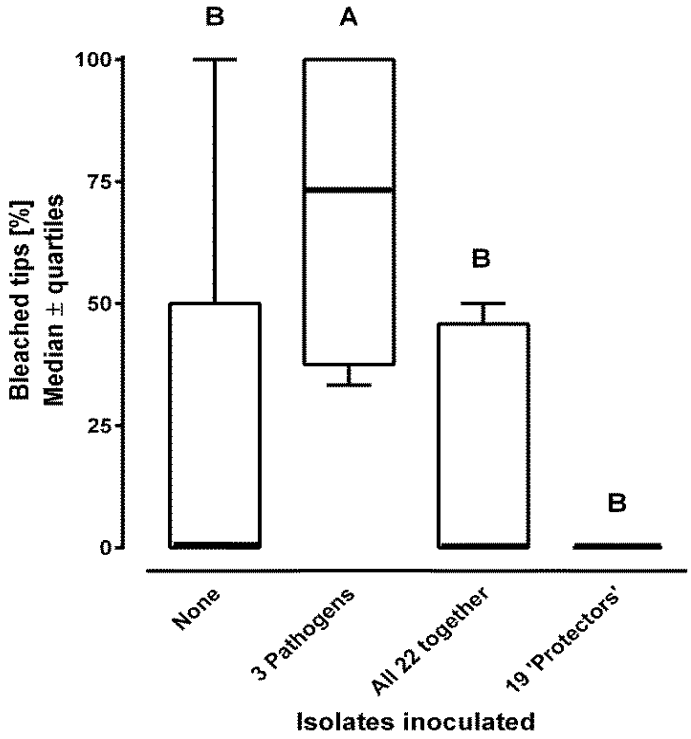
653

654 **Figures**




655

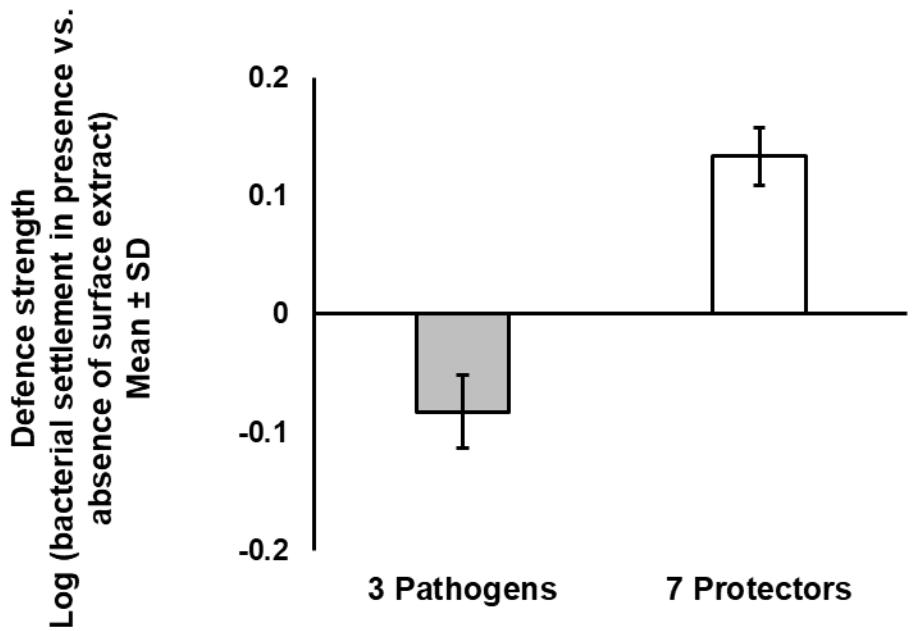
656 **Fig. 1:** Risk of thallus tip bleaching in *A. vermiculophyllum* after inoculation of 22 bacterial
657 strains relative to control thalli without such inoculation. Numbers of independent infection
658 experiments (each with n = 6) were three in the case of *K. algicida,* two in the cases of *C.*
659 *eckloniae* and *P. arctica* and one in all other cases. Only isolates that affected the risk at $p <$
660 0.05 are shown. Asterisks indicate isolates which were significantly pathogenic or protective
661 after Bonferroni-correction ($p<0.00086$). Error bars±95% CI.




662

663 **Fig. 2:** Relative amounts of bleached thallus tips in *A. vermiculophyllum* after inoculation
664 with 3 pathogenic bacterial isolates with 19 protective bacterial isolates, with all 22 isolates
665 together and in controls without any inoculation. Different letters indicate treatments that
666 are significantly different ($n = 12$; Kruskal-Wallis-ANOVA ($p < 0.0001$) and Dunn's post hoc
667 test ($p < 0.05$)). Pathogenic strains include both 'significant pathogens' and 'potential
668 pathogens'. Protective strains include both 'significant protectors' and 'potential
669 protectors'. Median ± quartiles.

670




671

672 **Fig. 3:** Mean anti-settlement activity of *Agarophyton* surface metabolites against three
673 pathogenic (one 'significant pathogen' and two 'potential pathogen') and seven protective
674 (five 'significant protector' and two 'potential protector') strains. Error bars ± SD (n=5);
675 Welch-corrected t-test, $p < 0.0001$.