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1 **Metabolic complementarity between a brown alga and associated**
2 **cultivable bacteria provide indications of beneficial interactions.**

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16 **Keywords: *Ectocarpus siliculosus*, symbiotic/mutualistic bacteria, genome-scale metabolic**
17 **networks, metabolic complementarity, holobiont**

18 **Abstract:**

19 Brown algae are key components of marine ecosystems and live in association with bacteria that are
20 essential for their growth and development. *Ectocarpus siliculosus* is a genetic and genomic model
21 for brown algae. Here we use this model to start disentangling the complex interactions that may
22 occur between the algal host and its associated bacteria. We report the genome-sequencing of 10
23 alga-associated bacteria and the genome-based reconstruction of their metabolic networks. The
24 predicted metabolic capacities were then used to identify metabolic complementarities between the
25 algal host and the bacteria, highlighting a range of potentially beneficial metabolite exchanges
26 between them. These putative exchanges allowed us to predict consortia consisting of a subset of
27 these ten bacteria that would best complement the algal metabolism. Finally, co-culture experiments
28 were set up with a subset of these consortia to monitor algal growth as well as the presence of key
29 algal metabolites. Although we did not fully control but only modify bacterial communities in our
30 experiments, our data demonstrated a significant increase in algal growth in cultures inoculated with
31 the selected consortia. In several cases, we also detected, in algal extracts, the presence of key
32 metabolites predicted to become producible via an exchange of metabolites between the alga and the
33 microbiome. Thus, although further methodological developments will be necessary to better control
34 and understand microbial interactions in *Ectocarpus*, our data suggest that metabolic
35 complementarity is a good indicator of beneficial metabolite exchanges in holobiont.

36 1 Introduction

37 Microbial symbionts are omnipresent and important for the development and functioning of
38 multicellular eukaryotes. Together the eukaryote hosts and their microbiota form meta-organisms
39 also called holobionts. Elucidating the interactions within microbial communities and how they affect
40 host physiology is a complex task and requires an understanding of the dynamics within the
41 microbiome and the host, as well as of possible inter-species interactions and/or metabolic exchanges
42 that could occur between the partners. One way to dissect those interactions is via targeted co-culture
43 experiments using culturable bacteria. This approach works particularly well for 1:1 or 1:2
44 interactions, but as the number of potentially interacting organisms increases, selecting the “right”
45 bacterial consortia becomes a major bottleneck (Lindemann *et al.* 2016).

46 Metabolic complementarity has previously been proposed as an indicator for potentially beneficial
47 host-symbiont interactions and can be assessed *in silico* using the metabolic networks of the host and
48 the microbiota (Dittami, Eveillard, *et al.* 2014; Levy *et al.* 2015). Common examples of metabolic
49 complementarity are associations of autotrophic and heterotrophic organisms such as corals and their
50 photosynthetic symbionts (Rohwer *et al.* 2002), or algae, and their heterotrophic bacterial biofilm
51 (Wahl *et al.* 2012). In this case, the autotrophic partner has a metabolic capacity (photosynthesis) that
52 allows for the production of metabolic intermediates (organic carbon), which can be further
53 metabolized by the heterotrophic partners. However, especially in systems with long-lasting
54 interactions more complex metabolic interdependencies are likely to evolve (*e.g.* Amin *et al.* 2015).

55 As a tool to further explore such interactions, Frioux *et al.* (Frioux *et al.* 2018) have proposed the
56 pipeline MiSCoTo. Given the metabolic networks of a host and several symbionts, this tool predicts
57 potential metabolic capacities of one partner that could be unlocked by a contribution of a metabolite
58 from another (*e.g.* the provision of carbohydrates by a photosynthetic organism unlocking the
59 biochemical processes related to primary metabolism in heterotrophs). Furthermore, this
60 computational approach uses these complementarities to define minimal consortia (*i.e.* with the
61 lowest possible number of exchanges/contributors) allowing the host to reach its maximum metabolic
62 potential. However, the actual predictive value of these models, both in terms of the effect on host
63 growth and fitness, and in terms of the metabolic scope (*i.e.* the metabolites producible by the
64 holobiont system), remains to be assessed.

65 Here we have applied the MiSCoTo tool to the filamentous brown alga *Ectocarpus siliculosus*, a
66 model filamentous brown alga with an available metabolic network (Prigent *et al.* 2014), as well as a
67 selection of 10 *Ectocarpus*-derived bacteria (KleinJan *et al.* 2017). We then selected specific minimal
68 microbial consortia for *in vivo* testing of the proposed hypotheses (growth rate, production of specific
69 metabolites). Our results demonstrate a clear positive effect of inoculation with the predicted
70 bacterial consortia on algal growth as well as an effect on the production of algal metabolites
71 predicted to depend on bacterial contributions. *In vivo* observations largely corresponded to *in silico*
72 predictions despite the incomplete input data (with models limited to annotated pathways) and the
73 fact that we had only limited control of the microbiome. The present work thus generates numerous
74 testable hypotheses on specific beneficial interactions between *Ectocarpus* and its microbiome, but
75 also provides a proof of concept for the overall predictive power of network-based metabolic
76 complementarity for beneficial host-microbe interactions.

77 2 Methods

78 2.1 Bacterial cultures and genome sequencing

79 Ten bacterial strains were selected from the 46 isolated by KleinJan et al. from *Ectocarpus subulatus*
80 (KleinJan et al. 2017). They were grown in liquid Zobell and/or diluted R2A until bacterial growth
81 was visible with the naked eye (~3 days at room temperature), and their identity was confirmed by
82 sequencing of the 16S rRNA gene with the primers 8F and 1492R (KleinJan et al. 2017). Bacterial
83 DNA was extracted using the UltraClean® Microbial DNA isolation kit (MoBio, Qiagen, Hilden,
84 Germany) and used for standard pair-end sequencing at the GENOMER platform (FR2424, Station
85 Biologique de Roscoff), using Illumina Miseq technology (V3 chemistry, 2x300bp). After cleaning
86 with Trimmomatic v0.38, default parameters (Bolger et al. 2014), the paired-end reads were
87 assembled using SPADes v3.7.0 (Bankevich et al. 2012; default parameters for long reads). The
88 RAST/SEED server (Aziz et al. 2008) was used for gene annotation, and sequences were later also
89 incorporated into the MAGE platform (Vallenet et al. 2006).

90 **2.2 In silico predictions of metabolic interactions and selection of consortia**

91 Bacterial metabolic networks were constructed using Pathway Tools version 20.5 (Karp et al. 2016)
92 and version 2 of the *Ectocarpus siliculosus* EC32 metabolic network for the host, prior to any gap-
93 filling step, in order to prevent the presence of possibly false positive reactions in the model.
94 (because these false positive reactions could hide algal bacterial interactions). This network
95 comprised a total of 2,118 metabolites, 1,887 metabolic reactions, and was able to produce five of the
96 50 metabolites known to be a part of the *Ectocarpus* biomass (Aite et al. 2018) with only the culture
97 medium as input. For the remaining 45 compounds the lack of producibility can be explained by the
98 presence of metabolic gaps – either because a reaction was missed during the reconstruction of the
99 network (missing annotation etc.), or because the corresponding pathways require metabolite
100 exchanges with other partners in the environment, e.g. bacteria. The more such gaps can be filled by
101 exchanging compounds between two metabolic networks, the higher we consider the degree of
102 metabolic complementarity between the corresponding organisms.

103 Here we used the MiSCoTo tool (Frioux et al. 2018) to compute such potential metabolic exchanges
104 between *Ectocarpus* and any of the ten targeted bacteria. The underlying model of MiSCoTo
105 assumes that a compound is producible by a host-symbiont community if there is a chain of
106 metabolic reactions which transforms the culture medium into the expected compound without taking
107 into consideration flux accumulations or competition for resources, and allowing for the exchange of
108 compounds across cell boundaries. These simplifications imply that compounds predicted to be
109 producible *in silico* may, in some cases, remain unproducible *in vivo*, although the consortium has all
110 the genes to activate the pathways.

111 In this study MiSCoTo was run twice, first to determine the scope of all algal compounds that
112 become producible via exchanges with all 10 bacterial genomes together, and as second time to select
113 minimal bacterial consortia for the production of these compounds. In both cases the Provasoli
114 culture medium was used as a source as defined previously (Prigent et al. 2014).

115 **2.3 Algal cultures**

116 Two of the six predicted bacterial consortia were tested experimentally via algal-bacterial co-culture
117 experiments. Additionally, each member of the two consortia was tested individually, as well one
118 other sequenced strain that was not part of any of the predicted minimal consortia, i.e. *Sphingomonas*
119 sp. 391. *Ectocarpus siliculosus* (strain 32; accession CCAP 1310/4, origin San Juan de Marcona,
120 Peru) was cultured under standard conditions (13 °C; 12h light regime) in Provasoli-enriched natural
121 seawater until the start of antibiotic treatment. Prior to co-culture experiments, algal filaments were
122 treated with a mixture of the following liquid antibiotics: 45 µg/ml Penicillin G, 22.5 µg/ml

123 streptomycin, and 4.5 µg/ml chloramphenicol dissolved in Provasoli-enriched artificial seawater 450
124 mM Na⁺, 532 mM Cl⁻, 10 mM K⁺, 6 mM Ca²⁺, 46 mM Mg²⁺, 16 mM SO₄²⁻. Filaments were exposed
125 to 25 ml of this solution for 3 days and then placed in Provasoli-enriched artificial seawater for 3
126 days to recover. The absence of bacteria on the algal surface was verified by microscopy using phase-
127 contrast (Olympus BX60, 1.3- PH3 immersion objective, 800x magnification) and by plating of algal
128 filaments on Petri dishes with Zobell medium followed by three weeks of incubation at room
129 temperature.

130 **2.4 Co-culture experiments**

131 For co-culture experiments, cell densities of bacterial cultures were determined using a BD FACS
132 CantoTM II flow cytometer (BD Bioscience, San Jose, CA) using samples fixed in Tris-EDTA.
133 Before the start of the experiment, antibiotic-treated algae (three replicate cultures per condition)
134 were inoculated with 2.3*10⁵ bacterial cells per strain and ml medium. Each co-culture was then
135 incubated for 4 weeks under standard algal growth conditions (see above). During this time, algal
136 growth was quantified by measuring the filament length of the algae each week using the binocular
137 microscope (3 measurements per replicate). Furthermore, bacterial abundance in the algal growth
138 medium was estimated using flow cytometry (described above) and bacteria attached to algal cell
139 walls were counted by microscopy (5x 10 µm long filaments observed per biological replicate, 800x
140 magnification in phase contrast). At the end of the experiment, general algal morphology was
141 observed using a LEICA DMi8 microscope and in parallel, remaining algal tissues were frozen in
142 liquid nitrogen and freeze-dried for downstream analyses. Two controls (three replicates each) were
143 run in parallel: a non-antibiotic treated positive control (CTRL w/o. ATB), and an antibiotic-treated
144 non-inoculated alga as a negative control (CTRL w. ATB).

145 **2.5 Bacterial community composition after co-culture experiments**

146 A metabarcoding approach was implemented to investigate the composition of the bacterial
147 community after the co-culture experiments. For each culture, 20 mg ground freeze-dried tissue
148 (TissueLyserII Qiagen, Hilden, Germany; 2x45sec, 30 Hz) was used for DNA extraction (DNeasy
149 Plant Mini Kit, Qiagen; standard protocol). Nucleotide concentrations were verified with
150 NanodropONE (ThermoFisher Scientific). A mock community comprised of DNA from 32 bacterial
151 strains (covering a variety of taxa) as well as a negative control were included in addition to the
152 samples (see Thomas *et al.* in prep. for details). Libraries were prepared according to the standard
153 Illumina protocol for metabarcoding MiSeq technology targeting the V3–V4 region (Illumina 2017)
154 and sequenced using Illumina MiSeq Technology (2x300 bp, pair-end reads; MiSeq Reagent v3 kit;
155 Platform de Séquencage-Génotypage GENOMER, FR2424, Roscoff).

156 Resulting raw sequences (7,354,164 read pairs) were trimmed using fastq_quality_trimmer from the
157 FASTX Toolkit (quality threshold 30; minimum read length 200) and assembled into 6,804,772
158 contigs using PandaSeq v2.11 (Masella *et al.* 2012). Data were analyzed with Mothur (V.1.40.3)
159 according to the MiSeq Standard Operating Procedures (Kozich *et al.* 2013). Contigs were pre-
160 clustered (allowing for four mismatches), and aligned to the Silva_SEED 132b database for sequence
161 classification. Chimeric sequences were removed (Vsearch) and the remaining sequences classified
162 taxonomically (Wang *et al.* 2007). Non-bacterial sequences were removed and the remaining
163 sequences were then clustered into operational taxonomic units (OTUs) at a 97% identity level and
164 each OTU was classified to the genus level where possible (Wang *et al.* 2007). All OTUs with n ≤ 10
165 sequences were removed resulting in a final data matrix with 1,834,992 sequences. The OTU matrix
166 was subsampled to have the same number of sequences per sample for downstream analyses.

167 **2.6 Targeted metabolomics**

168 Seven metabolites predicted to be producible by the algae only in presence of metabolic exchanges
169 with specific bacteria were selected for targeted metabolite profiling after manual verification of
170 automatic predictions of corresponding pathways in the algal and bacterial networks and based on
171 their biological importance for the alga: L-histidine, putrescine, beta-alanine, nicotinic acid, folic
172 acid, auxin, and spermidine. Metabolites were extracted from 10 mg of ground, freeze-dried tissue
173 using a triple extraction protocol based on the method of Bligh and Dyer (1959): two ml of
174 methanol:chloroform:water (6:4:1) were used as first extraction solvent, then the remaining pellet
175 was extracted with 1 ml of chloroform:methanol (1:1), and finally, a 3rd extraction was performed
176 using 1ml of H₂O. The supernatants of each extraction were pooled and evaporated under a stream of
177 nitrogen. The residue was then resuspended in 100 µl methanol:water (1:1) and analyzed on an
178 ACQUITY Ultra-performance convergence chromatography (UPC²) system (Waters®, Milford,
179 USA) equipped with a Viridis BEH column (3x100 mm, 1.7 µm). A linear gradient of two solvents
180 was used to separate peaks: supercritical carbon dioxide (Solvent A), and methanol spiked with 0.1%
181 formic acid (Solvent B). The gradient ran from 5% to 25% of solvent B (35% for spermidine and
182 nicotinic acid) during 2 minutes, was kept at this level for another 2 minutes and then gradually
183 reduced back to 5% during 3 minutes. The UPC² system was coupled to a Xevo G2 Q-Tof mass
184 spectrometer (Waters), operating in positive ESI ion mode (m/z 20–500). Blanks, as well as
185 standards of all 7 compounds obtained from Sigma-Aldrich (St. Louis, MO, USA), were run in
186 parallel to samples. The resulting chromatograms were then used to examine the presence/absence of
187 the target compounds in the other samples based on retention time and the mass spectra. Analyses
188 were performed at the METABOMER platform (FR2424, Station Biologique de Roscoff).

189 **2.7 Statistical analyses**

190 Growth data (both algal and bacterial) were confirmed to follow a normal distribution using a
191 Shapiro-Wilk test (Rstudio v1.0.44). Significant differences between all treatments after four weeks
192 of co-culture (day 28) were calculated with an ANOVA and a Tukey honestly significant difference
193 (HSD) post-hoc test with a significance level α 0.05 using the PAST software version 3.20 (Hammer
194 *et al.* 2001).

195 **3 Results**

196 **3.1 Predicted metabolic interactions and selection of beneficial bacterial consortia**

197 Genome sequencing and subsequent bioinformatics analyses yielded bacterial genome assemblies
198 with sufficient coverage and 11-72 scaffolds per genome (Table 1). Metabolic networks were then
199 reconstructed for these ten genomes. On average, 1,714 reactions, 111 transport reactions, and 1,405
200 metabolites (Table 2) were predicted per bacterium. These reactions belonged, again on average, to
201 261 pathways, 137 of which were complete and 124 were incomplete (*i.e.* missing one or more
202 reactions). Based on metabolic complementarity analysis carried out using MiSCoTo, these bacterial
203 networks were predicted to enable the production of 160 additional compounds with the algal
204 networks, including several polyamines (Cadaverine, Spermidine, Agmatine), amino acids (Histidine,
205 Tyrosine, beta-alanine), vitamins B3, B9, and E, several lipids and lipid derivatives, and nucleic
206 acids. Please refer to Supplementary Table S1 for a complete list of compounds. Many of these
207 compounds were also previously predicted via the metabolic interaction between the same strain of
208 *E. siliculosus* and the associated bacterium *Candidatus* Phaeomarinobacter ectocarpi (Dittami,
209 Barbeyron, *et al.* 2014; Prigent *et al.* 2017). A total of six bacterial consortia comprising three
210 bacterial strains each (Table 3) were predicted to be sufficient to enable the production of all of these

211 compounds. Of these six proposed consortia, two comprising one phylogenetically distinct bacterium
212 each (i.e. the *Bacterioidetes Imperialibacter* vs the *Gammaproteobacterium Marinobacter*) were
213 chosen for *in vivo* testing using algal-bacterial co-cultures.

214 **3.2 Growth rates in co-culture experiments**

215 The inoculation with one or several bacterial strains significantly enhanced algal growth by a factor
216 of 2 compared to controls (Figure 1A). This positive effect was observed both for the predicted
217 bacterial consortia and for all the individual strains tested. At the same time, the abundance of
218 bacteria on algal filaments after four weeks of cultivation was significantly lower in cultures initially
219 inoculated with bacteria compared to both controls with and without initial antibiotic treatment
220 (Figure 1B), although bacterial cell counts in the medium were similar between co-culture
221 experiments and the non-inoculated control after 28 days (Supplementary Figure S1).

222 **3.3 Bacterial impact on morphology**

223 Compared to the negative control, which exhibited a ball-like morphology typical for “axenic”
224 cultures (Tapia *et al.* 2016), all bacterial inocula tested resulted in filamentous thalli with clear
225 branching patterns (Figure 2). We furthermore observed differences in the branching patterns
226 depending on the bacterial inocula. For example, *Sphingomonas*-inoculated cultures produced
227 relatively long filaments with few branching sites (Figure 2H), whereas *Hoeflea*-inoculated cultures
228 produced filaments with frequent branching (Figure 2E). *Imperialibacter* induced aggregation of
229 individual filaments (Figure 2F), while in all other co-cultures, filaments remained more or less
230 separated. These differences were, however, difficult to quantify given complexity of their
231 morphology.

232 **3.4 (Algal) metabolome in co-culture conditions**

233 Seven putatively key metabolites (l-histidine, putrescine, beta-alanine, nicotinic acid, folic acid,
234 auxin, and spermidine) predicted to be non-producible by the alga alone but producible via exchanges
235 with some bacterial consortia, were quantified in algal tissues by UPC²-MS after four weeks of co-
236 culture. The presence/absence of these metabolites is shown in Figure 3, comparing both the
237 predicted producibility by metabolic network analysis and the experimental UPC²-MS results. In the
238 negative control, i.e. antibiotic-treated algae that were not inoculated with bacteria, none of the
239 compounds could be identified by UPC²-MS confirming the computational predictions. In contrast,
240 in all co-cultures, at least one target compound was experimentally detected. Furthermore, each
241 compound became producible in at least one of the co-cultures. Overall, across the 56 predictions
242 made based on the metabolic networks (7 metabolites x 8 consortia including the individual bacteria
243 and the negative control) *in silico* and *in vivo* data agreed in 28 cases (Figure 3). Only in four cases
244 did we observe the presence of a metabolite although it was not predicted by the networks. Finally, in
245 24 cases we did not detect the presence of a metabolite predicted to be producible in the co-cultures.

246 **3.5 Bacterial community composition after co-culture experiments**

247 The bacterial community composition of each sample was analyzed by 16S rDNA metabarcoding at
248 the end of the co-culture experiments. This was done to verify if the bacteria inoculated had grown in
249 the co-cultures and to determine to what extent other bacteria were present and affected by the
250 inoculations. The results (Table 4) show that, except for *Imperialibacter*, all of the bacterial strains
251 inoculated were detected in the corresponding co-cultures 28 days after inoculation. However, except
252 for *Marinobacter* and *Hoeflea*, read abundances of these strains were low compared to the total

253 number of reads. In parallel, several other OTUs that had not been inoculated were detected in our
254 co-culture experiments, suggesting that these bacteria were at least partially resistant to or protected
255 from (*e.g.* within the cell wall) the antibiotic treatments applied, and were able to recover under the
256 experimental conditions: in total 30 additional OTUs with a minimal abundance of 1% of total reads
257 were detected in our samples, accounting for 63 to 82% of the total reads. Furthermore, *Hoeflea* reads
258 were dominant in all samples including the non *Hoeflea*-inoculated cultures (14-30% of total reads).

259 **4 Discussion**

260 **Metabolic complementarity, a powerful metric despite limitations**

261 Metabolic complementarity intuitively seems like an excellent marker for beneficial metabolic
262 interactions. The more organisms are complementary at the metabolic and by extension the gene
263 level, the more they can potentially benefit from each other (Levy *et al.* 2015); the more they overlap
264 in terms of metabolic pathways, the more likely they are to compete for the same resources (Kreimer
265 *et al.* 2012). There are, however, two important restrictions that limit the applicability of this simple
266 idea. First, the possibility of a beneficial exchange does not necessarily mean that it will occur,
267 because this may require the presence and activation of excretion/uptake mechanisms in both
268 partners, *e.g.* via chemical or environmental cues. Secondly, the genome-scale metabolic models used
269 to predict metabolic complementarities may be partially erroneous and incomplete. For instance,
270 metabolic networks frequently do not comprise interactions of chemical signals with receptor
271 molecules, which may be key to regulate interactions (Zhou *et al.* 2016; Wang *et al.* 2018).
272 Furthermore, in many cases, they are based on automatic predictions and annotations of protein
273 sequences, which may, in some cases, miss genes or introduce overpredictions of functions (Schnoes
274 *et al.* 2009). In this paper, we provide first *in vivo* tests of host-microbe interactions inferred from
275 genome-based predictions of metabolic complementarity. Despite the aforementioned restrictions and
276 simplifications, our results discussed below provide a strong indication that, genome-based
277 predictions of metabolic complementarity is a powerful tool to handle the complexity of host microbe
278 systems and to generate hypotheses on their interactions.

279 **Similar complementarities found across studies and *Ectocarpus* symbionts.**

280 Compared to a previous analysis of metabolic complementarity between *Ectocarpus* and another
281 associated bacterium, *Candidatus Phaeomarinobacter ectocarpus*, (Dittami, Barbeyron, *et al.* 2014;
282 Prigent *et al.* 2017), newly producible compounds predicted in this study were largely similar,
283 notably regarding polyamines, histidine, beta-alanine, and auxin. This similarity persists even though
284 metabolic complementarity analyses were performed using MiSCoTo, which incorporates the notion
285 of different compartments minimizing the number metabolite exchanges (Frioux *et al.* 2018) and
286 despite the fact that different bacteria were examined. The main difference compared to the previous
287 study is that numerous additional compounds were predicted to be exchanged, which can be
288 explained by the fact that ten rather than one bacterial network were available to complete the algal
289 network.

290 **Inoculation with metabolically complementary bacteria enhances growth rate and impacts** 291 **morphology and metabolism**

292 As described above, both the bacterial consortia tested, as well as all of the bacteria inoculated
293 individually had clear positive effects on algal growth and impacted algal morphology and metabolite
294 profiles, even though, by the time the co-cultures were harvested, some of the inoculated bacteria
295 were present only in very low abundance or even below the detection limit. These positive effects

296 could be due either to interactions early in the co-culture experiments followed by a decline in
297 bacterial abundance, or due to the capacity of bacteria to impact and interact with their algal hosts
298 even at very low cell concentrations. The latter would support the hypothesis that part of the
299 observed effects may not be due to the exchanges of (abundant) primary metabolites, such as the
300 predicted histidine/histidinol, but due to lowly concentrated signaling molecules or growth hormones.
301 One such compound could be the examined auxin, which was detected in 5 of the 7 tested co-
302 cultures, and which has previously been shown to modify the developmental patterns and
303 morphology of *Ectocarpus* cultures (Le Bail *et al.* 2010) in a similar way as bacterial inoculations.
304 Another observation was that the abundance of bacteria on algal filaments but not in the medium was
305 significantly lower in co-culture conditions compared to the controls. This suggests that the
306 inoculated bacteria, either directly, or indirectly, by stimulating algal growth or defense, can also
307 regulate biofilm formation (see Goecke *et al.* 2010 for a review).

308 Interestingly, although differences in the effects of individual bacteria and bacterial consortia were
309 observed on metabolite profiles and morphology, all consortia had similar effects on algal growth.
310 Indeed, all of the tested bacteria, including *Sphingomonas*, which was not part of the minimal
311 solutions proposed by MiSCoTo, were to a great extent complementary to the alga, already covering
312 a large part of the metabolic gaps. In future studies, it may be particularly useful to incorporate a
313 larger range of bacteria, possibly from other sources so that they are not expected to have evolved
314 mutualistic interactions with brown algae. These negative controls could then be used to correlate
315 growth rates with the presence or absence of specific metabolic capacities in the network. Once the
316 list of candidate metabolite exchanges has been narrowed down by such comparisons, supplying
317 these metabolites from artificial sources but also testing for their excretion into the medium by
318 bacteria can be used to corroborate their role.

319 **Predicted metabolic exchanges likely to occur in part**

320 With respect to the predictions of target metabolites, we observed that for a large number of cases,
321 predictions from the metabolic networks corresponded to the observations made by experimental
322 metabolic profiling: none of the target metabolites were detected in the negative control, and only in
323 four cases (Figure 3), did we detect compounds in co-cultures that were not predicted to be there.
324 This could either be attributed to undetected metabolic pathways in the examined/added bacteria (*e.g.*
325 due to missing annotations) or, more likely, to the activity of other bacteria present in our co-culture
326 experiments (see below). Furthermore, there were several cases in which a potentially co-producible
327 metabolite was not detected in our co-cultures. Here two explanations appear particularly likely: first,
328 the metabolites in question may be produced but quickly metabolized in certain consortia, so that
329 they do not accumulate sufficiently to be detectable in our cultures; secondly, it is possible that the
330 corresponding biosynthetic pathway of the metabolite was not active or that the necessary exchange
331 of metabolites was not taking place. To resolve this point in future experiments, the addition of gene
332 expression data may help to establish whether or not biosynthetic or degradation pathways are active.
333 From a global perspective, however, the fact that none of the compounds in question were detected in
334 negative controls, but all of them it at least one co-culture condition, constitutes a highly promising
335 result.

336 **Outlook**

337 In our opinion, the main challenge for future *in vivo* studies of metabolic complementarity will be to
338 better control the *Ectocarpus*-associated microbiome in co-culture experiments, and thus to avoid any
339 impact of non-inoculated microbes. The currently applied antibiotic treatments are successful in

340 removing bacteria from the algal surface to a level where they are no longer detectable by
341 microscopy and spreading on culture medium, but once the treatment is stopped and algae are left to
342 recover, so do parts of the microbiome, possibly from spores that were inactive or embedded in the
343 algal cell wall and thus less susceptible to our treatments (Tetz and Tetz 2017). In the light of these
344 results, we strongly recommend routine metabarcoding analysis for any type of coculture experiment,
345 also in other model systems. One possibility in the future would be to use axenization protocols
346 based on the movement of gametes, as has been done for *Ulva mutabilis* (Spoerner *et al.* 2012); at
347 least some strains of *Ectocarpus* have previously been shown to produce phototactic gametes (Kawai
348 *et al.* 1990). A second alternative is the continuous use of antibiotics throughout the experiment, and
349 working with antibiotic-resistant bacterial strains. In this context a better understanding of the
350 metabolic requirements of the algae will help to durably maintain axenic cultures.

351 Despite these challenges, the present study constitutes an important proof of concept for the use of
352 metabolic complementarity to study simplified system of mutualistic host-symbiont interactions. We
353 anticipate that, in the long run, this concept can be applied not only to controlled co-culture
354 experiments, but that it will also prove useful for the interpretation of more complex datasets such as
355 metatranscriptomic or metagenomic data.

356 **5 Conflict of Interest**

357 The authors declare that the research was conducted in the absence of any commercial or financial
358 relationships that could be construed as a potential conflict of interest.

359 **6 Author Contributions**

360 Conceived the experiments: BB, HK, SD; Conceived in silico analyses: CF, AS. Performed
361 experiments: BB, HK; Performed analyses: BB, ALS, EF, CF, MW, SD, EC, CL; Wrote the
362 manuscript: SD, HK, BB. Corrected and approved of the final manuscript: all authors.

363 **7 Funding**

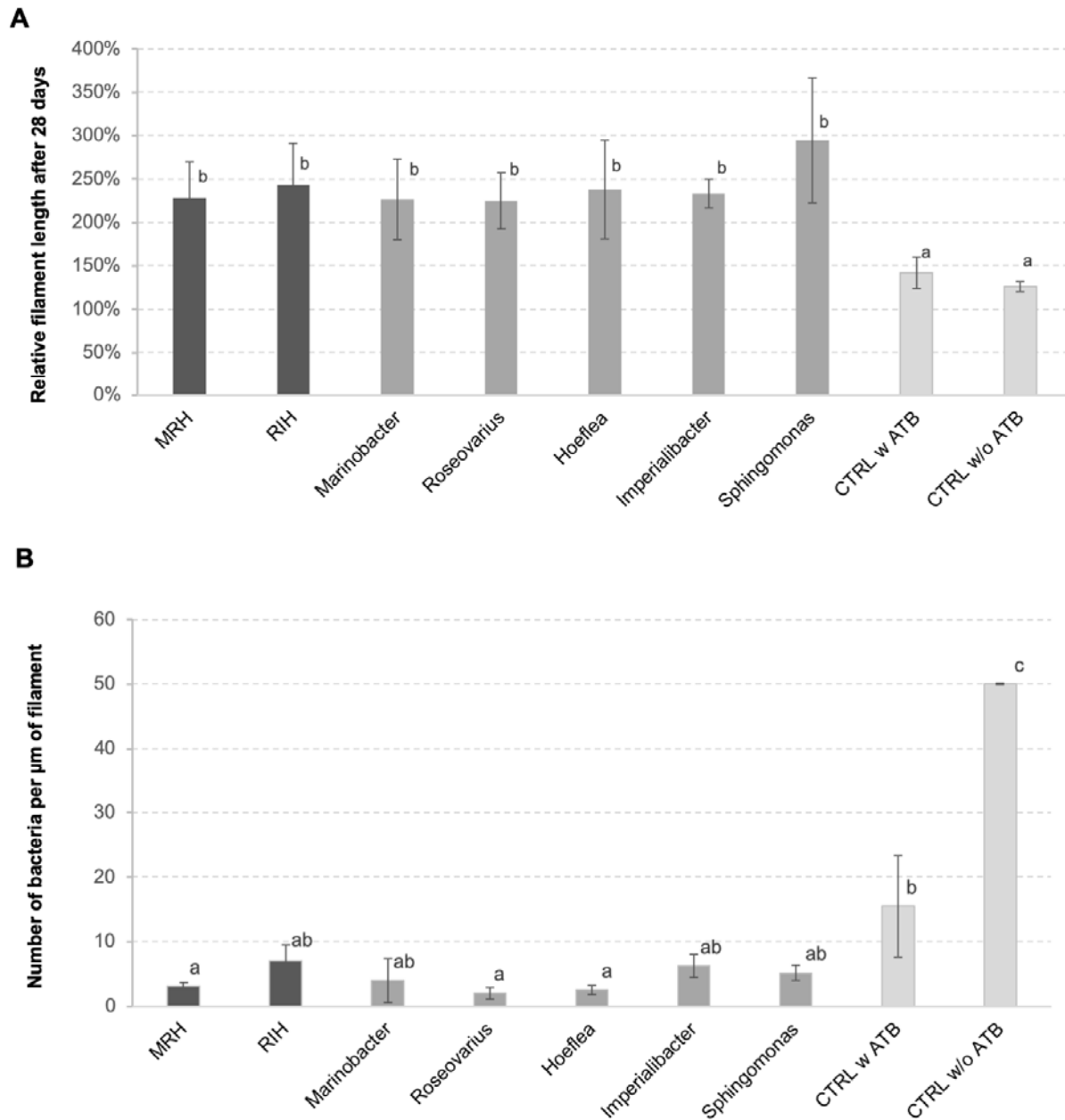
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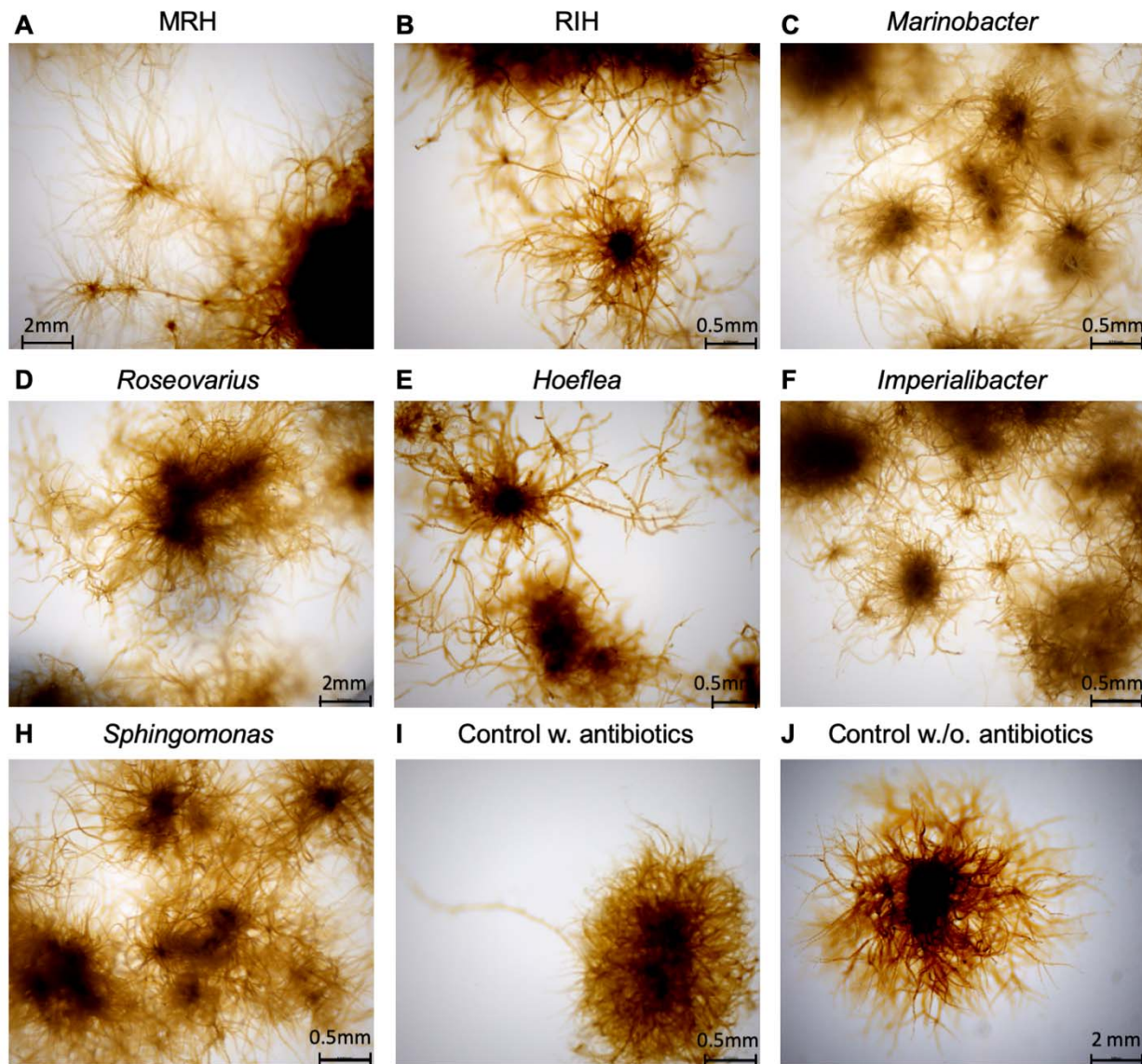
380 10-INBS-09 and ANR-11-INBS-0013) are acknowledged for support within the MicroScope
381 annotation platform.

382 **9 Figures**



383

384 **Figure 1:** A) Relative length of *E. siliculosus* filaments after 28 days of (co-)culture compared to the
385 starting point. B) Number of bacteria detected on algal filaments after 28 days of co-culture. Both
386 panels A and B show means of 3 replicate co-cultures \pm SD and differences are statistically
387 significant (one-way ANOVA $p < 0.01$). The letters above the columns indicate the results of a
388 TUKEY HSD pairwise comparisons ($p < 0.05$). CTRL = control, ATB = antibiotic treatment, MRH =
389 *Marinobacter-Roseovarius-Hoeflea*; RIH = *Roseovarius-Imperialibacter-Hoeflea*.



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Figure 2: Morphological effect co-cultures with bacteria on *E. siliculosus* after 4 weeks of co-culturing. MRH = *Marinobacter-Roseovarius-Hoeflea*, RIH = *Roseovarius-Imperialibacter-Hoeflea*.

Brown algal-bacterial metabolic interactions

Target	Exchange	MRH	RIH	Marinobacter	Roseovarius	Hoeflea	Imperialibacter	Sphingomonas	CTRL w ATB
Spermidine	Dehydrospemidine	+/-	+/-	+/-	+/+	+/-	+/-	+/-	-/-
Putrescine	Agmatine	+/-	+/+	+/+	+/+	+/-	+/-	+/-	-/-
Nicotinic acid	Nicotinic acid	+/-	+/+	+/-	+/-	+/-	-/+	+/-	-/-
Folic acid	dihydrofolate	+/-	+/-	-/-	-/-	-/-	-/-	-/+	-/-
Auxine	Indole-3-acetaldehyde	+/+	+/+	-/-	-/-	-/+	+/+	-/+	-/-
L-histidine	Histidinol	+/-	+/-	+/-	+/-	+/+	+/+	+/+	-/-
Beta-Alanine	3-ureidopropanoate	+/-	+/+	+/+	+/-	+/+	+/-	-/-	-/-

394

395 **Figure 3:** Comparison of predicted production of target metabolites in co-cultures based on
 396 metabolic networks (symbol before the slash) and results from targeted UPC²-MS analyses of algal
 397 filaments after 28 days (symbol after the slash). The column “Exchange” indicates one possible
 398 compound provided by the microbiome leading to the production of the compound in the column
 399 “Target” in the algal metabolome; it was these target metabolites that were tested for using UPC²-
 400 MS. All experiments were carried out in triplicate, each replicate of the same condition yielding
 401 identical results. (-): a target metabolite was not predicted/detected (+): a metabolite was
 402 predicted/detected. Green highlights conditions where predictions correspond to the in vivo
 403 observations, red highlights compounds that were detected although no pathway was predicted.
 404 Yellow indicates compounds potentially producible via bacterial exchanges that were not detected.
 405 MRH: *Marinobacter-Roseovarius-Hoeflea*; RIH: *Roseovarius-Imperialibacter-Hoeflea*; CTRL =
 406 control; ATB = antibiotic treatment.

407

408 10 Tables

409 **Table 1:** Overview of bacterial genomes used in this study and corresponding assembly statistics.

	raw reads	# scaffolds	genome size (mbp)	N50 (mbp)	Coverage	mapped reads
<i>Bosea</i> sp. 5A	1 863 417	26	6.34	0.98	133 X	99.91%
<i>Erythrobacter</i> sp. 430	1 065 278	11	3.14	0.44	157 X	99.93%
<i>Hoeflea</i> sp. 425	3 734 649	41	5.22	1.26	326 X	99.94%
<i>Imperialibacter</i> sp. R6	1 553 981	65	6.8	0.21	111 X	99.94%
<i>Marinobacter</i> sp. HK15	1 587 675	14	4.39	1.11	172 X	99.93%
<i>Rhizobium</i> sp. 404	1 332 560	27	4.2	0.45	148 X	99.93%
<i>Roseovarius</i> sp. 134	987 463	73	4.68	0.18	150 X	99.92%
<i>Roseovarius</i> sp. 420	803 175	85	4.68	0.12	79 X	99.89%
<i>Sphingomonas</i> sp. 631	1 111 277	25	3.28	0.29	150 X	99.87%
<i>Sphingomonas</i> sp. 391	1 150 343	74	4.6	0.16	113 X	99.91%

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413 **Table 2:** Predicted metabolic pathways (complete pathways in parentheses), reactions and
 414 metabolites in bacterial metabolic networks.

	number of pathways	number of reactions	transport reactions	number of metabolites
<i>Bosea</i> sp. 5A	298 (187)	1892	153	1557
<i>Erythrobacter</i> sp. 430	218 (91)	1532	63	1247
<i>Hoeflea</i> sp. 425	315 (170)	1920	129	1558
<i>Imperialibacter</i> sp. R6	239 (131)	1711	100	1425
<i>Marinobacter</i> sp. HK15	249 (128)	1679	128	1364
<i>Rhizobium</i> sp. 404	289 (142)	1814	125	1462
<i>Roseovarius</i> sp. 134	263 (146)	1703	125	1418
<i>Roseovarius</i> sp. 420	263 (143)	1701	125	1418
<i>Sphingomonas</i> sp. 361	224 (108)	1519	69	1239
<i>Sphingomonas</i> sp. 391	254 (126)	1671	92	1358

415

416 **Table 3:** Minimal bacterial consortia predicted by MiSCoTo that enabled the production of 160 algal
 417 compounds. See Supporting table S1 for a detailed list of compounds.

Solution proposed by MiSCoTo	In vivo testing?
<i>Marinobacter</i> sp. HK15, <i>Roseovarius</i> sp. 420, <i>Hoeflea</i> sp. 425	Yes
<i>Roseovarius</i> sp. 420, <i>Imperialibacter</i> sp. R6, <i>Hoeflea</i> sp. 425	Yes
<i>Marinobacter</i> sp. HK15, <i>Bosea</i> sp. 5a, <i>Roseovarius</i> sp. 420	No
<i>Marinobacter</i> HK15, <i>Hoeflea</i> sp. 425, <i>Roseovarius</i> sp. 134	No
<i>Imperialibacter</i> sp. R6, <i>Hoeflea</i> sp. 425, <i>Roseovarius</i> sp.134	No
<i>Marinobacter</i> sp. HK15, <i>Bosea</i> sp. 5a, <i>Roseovarius</i> sp. 134	No

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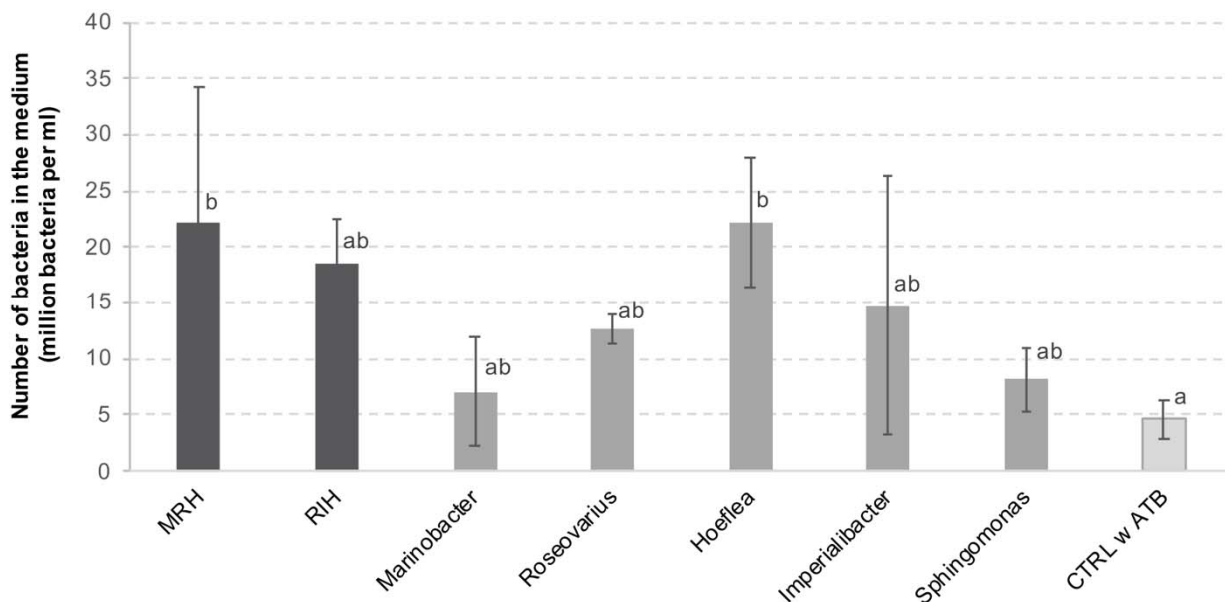
420 **Table 4:** Observed abundance of target OTUs after four weeks of co-culture. The table shows
 421 number of reads obtained corresponding to each OTU (mean three replicates \pm SD). Bold numbers on
 422 grey background indicate OTUs expected to be present based on the inoculations.

	MRH	RIH	<i>Marino- bacter</i>	<i>Roseo- varius</i>	<i>Hoef- lea</i>	<i>Im- periali- bacter</i>	<i>Sphing- omonas</i>	CTRL w. ATB	CTRL w./o. ATB
<i>Marinobct.O</i> TU00030	82 \pm 48	0 \pm 0	1103 \pm 1068	0 \pm 0	0 \pm 0	0 \pm 0	38 \pm 38	0 \pm 0	1 \pm 1
<i>Roseovarius</i> OTU00055	8 \pm 7	11 \pm 3	1 \pm 1	41 \pm 10	1 \pm 1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
<i>Hoeflea</i> OTU00001	10265 \pm 1586	7644 \pm 889	4483 \pm 2777	15635 \pm 1349	15321 \pm 3515	13426 \pm 5338	10216 \pm 4345	8899 \pm 2811	3618 \pm 1055
<i>Imperialib.</i> OTU00044	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
<i>Sphingomn.</i> OTU00097	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	1 \pm 1	4 \pm 4	0 \pm 0	0 \pm 0
Other OTUs*	39403 \pm 2138	23458 \pm 1828	26223 \pm 3187	36374 \pm 7810	34190 \pm 5508	38076 \pm 4292	29066 \pm 3302	42323 \pm 9670	28009 \pm 5897

423 * see Supplementary Figure S2 for details

424 11 Supplementary Material

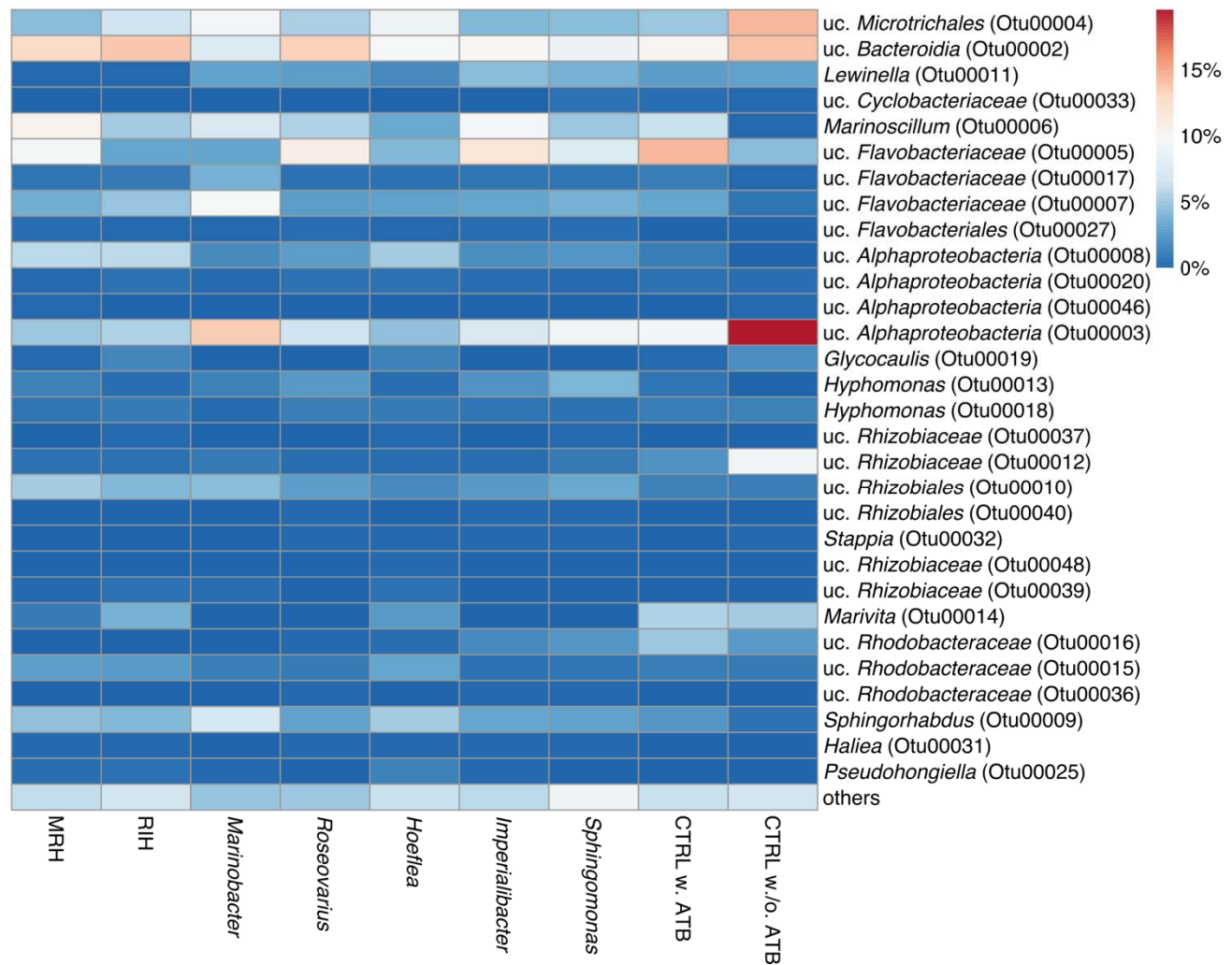
425 **Supplementary Table S1:** Metabolites predicted to become producible by the alga as a result of
 426 metabolite exchanges between the alga and bacteria. (uploaded separately)



427

428 **Supplementary Figure S1:** Number of bacteria detected in the algal culture medium after 28 days of
 429 co-culture. The graph shows means of 3 replicates \pm SD and differences are statistically significant
 430 (one-way ANOVA $p < 0.01$). The letters above the columns indicate the results of a TUKEY HSD
 431 pairwise comparisons ($p < 0.05$). CTRL = control, ATB = antibiotic treatment.

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432

433 **Supplementary Figure S2:** Heatmap of relative OTU abundance for all 30 OTUs that made up over
 434 1% of the total number of reads and that were not inoculated (See Table 4 for the latter). This
 435 heatmap as generated using the ClustVis service (Metsalu and Vilo 2015) using “correlation” as a
 436 distance measure and “average linkage” as clustering method. The color code corresponds to the
 437 mean sequence abundance for each OTU in the three replicates a percentage of total reads; uc. =
 438 unclassified

439

440

441 **12 Data Availability Statement**

442 The metabarcoding data generated for this study has been deposited at the European Nucleotide
443 Archive (ENA) under project accession number PRJEB34356. The bacterial genomes have been
444 deposited at the ENA under the sample accessions ERZ1079053-ERZ1079062.

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