

# Bacteria-induced morphogenesis of *Ulva intestinalis* and *Ulva mutabilis* (Chlorophyta)

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1 **Bacteria-induced morphogenesis of *Ulva intestinalis* and *Ulva mutabilis* (Chlorophyta): a**  
2 **contribution to the lottery theory.**

3

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8

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10 ***Microbacterium*, morphogenesis.**

11

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25 **Abstract:**

26 The green marine macroalgae of the class Ulvophyceae (Ulvophytes) are common algae  
27 distributed worldwide particularly in intertidal areas, which play a key role in aquatic  
28 ecosystems. They are potentially valuable resources for food, animal feed and fuel but can also  
29 cause massive nuisance blooms. Members of Ulvaceae, like many other seaweeds, harbour a rich  
30 diversity of epiphytic bacteria with functions related to host growth and morphological  
31 development. In the absence of appropriate bacterially-derived signals, germ cells of the genus  
32 *Ulva* develop into “atypical” colonies consisting of undifferentiated cells with abnormal cell  
33 walls. This paper examines the specificity of bacteria-induced morphogenesis in *Ulva*, by cross-  
34 testing bacteria isolated from several *Ulva* species on two *Ulva* species, the emerging model  
35 system *Ulva mutabilis* and the prominent biofouler species *Ulva intestinalis*. We show that pairs  
36 of bacterial strains isolated from species other than *U. mutabilis* and *U. intestinalis* can fully  
37 rescue axenic plantlets generated either from *U. mutabilis* or *U. intestinalis* gametes. This  
38 laboratory-based study demonstrates that different compositions of microbial communities with  
39 similar functional characteristics can enable complete algal morphogenesis and thus supports the  
40 “competitive lottery” theory for how symbiotic bacteria drive algal development.

41

42

43 **Introduction:**

44 Macroscopic marine algae (seaweeds) are significant primary producers in the oceans, which  
45 cover about 71% of earth's surface. Seaweeds are known as 'ecosystem engineers' due to their  
46 critical roles in marine environments, where they modulate the supply of resources to other  
47 species and alter the physical state of the surrounding environment, including sediments and  
48 water flow (Jones *et al.*, 1994; Alongi 1998). Seaweeds are important for maintaining local  
49 biodiversity (Schiel *et al.*, 2007), create a protective environment for numerous invertebrate  
50 species (Wilson *et al.*, 1990; Bulleri *et al.*, 2002) and provide an essential habitat for a range of  
51 epibionts, from microscopic organisms to macroinvertebrates (Fraschetti *et al.*, 2006). However,  
52 seaweeds can also cause significant nuisance blooms due to eutrophication in shallow coastal  
53 areas, which are detrimental to the environment and can harm ecosystems (Smetacek *et al.*,  
54 2013). In a commercial context, there is increasing interest in the use of marine biomass  
55 worldwide with multiple traditional and novel applications in food, fuel, high-value chemical and  
56 pharmaceutical industries and also in aquaculture, which is one of the promising market sectors  
57 (Kraan 2013).

58 There is growing interest in defining macroalgae-associated bacterial communities and  
59 macroalgal development and morphogenesis (Charrier *et al.*, 2017). A number of studies have  
60 shown that different species of seaweeds growing in the same ecosystem are associated with  
61 species-specific bacterial strains (Lachnit *et al.*, 2009; Barott *et al.*, 2011; Lachnit *et al.*, 2011),  
62 leading to the hypothesis that the association between microorganisms and algae is host-specific.  
63 This assumption is supported by observations that a significantly different phylum composition  
64 of bacteria was associated with each of three co-existing algae sampled at regular intervals over  
65 two years (Lachnit *et al.*, 2011). Moreover, the same species of seaweeds growing in different  
66 ecological habitats can associate with similar bacterial species (Lachnit *et al.*, 2009). Although it  
67 has been suggested that the bacterial-algal association is determined by the algal host (Longford  
68 *et al.*, 2007), bacterial isolates from seaweeds can vary with season and host life-cycle stage  
69 (Lachnit *et al.*, 2011) and even different tidal pools in close proximity (Burke *et al.*, 2011a). It  
70 was also reported by Cray *et al.*, (2013) that the pre-eminence of some species e.g.  
71 *Proteobacteria* and *Firmicutes* is the result of their ability to compete with other species due to

72 (i) high resistance to various stress factors (ii) existence of different pathways for generating  
73 energy.

74 In contrast, based on a large-scale sequencing analysis, Burke *et al.* (2011b) suggested “the  
75 competitive lottery model” for algal microbiomes, originally developed by Sale (1976) for  
76 explaining the coexistence of reef fish species in the same niche. They propose that different  
77 microbial communities with similar functional characteristics (defined by the genes present in  
78 the microbial genomes) can occupy the same algal species. Different microbiomes were isolated  
79 from different *Ulva australis* Areschoug samples in the same niche space and at different times  
80 in the year. The “competitive lottery” model states that the structuring of microbial communities  
81 on the surface of host algae is controlled by the presence of particular microbial functional genes  
82 rather than microbial taxonomic entities (Burke *et al.*, 2011b). It is suggested that these functions  
83 are related to the ecophysiological roles of alga-associated microbial communities in general, i.e.  
84 detecting and moving towards the host, followed by attaching to the host and forming a biofilm,  
85 then responding to host environmental factors (Burke *et al.*, 2011a; Friedrich 2012). This  
86 functional assistance would result in formation of a holobiont, an entity composed of an alga  
87 with its associated functionally important bacteria (Egan *et al.*, 2011).

88 Growth and morphogenesis of various species of the green macroalga *Ulva* such as *U.*  
89 *mutabilis*, *U. pertusa*, *U. linza* and *U. fasciata* can be controlled by a variety of marine bacterial  
90 species including members of the *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Provasoli,  
91 1958; Fries 1975; Nakanishi *et al.* 1996; Matsuo *et al.* 2003; Marshall *et al.* 2006; Sing *et al.*  
92 2011; Spoerner *et al.* 2012; Wichard 2015). Marshall *et al.*, (2006) assessed the effects of 38  
93 unique bacterial strains, isolated from three species of *Ulva*, on the growth rate and  
94 morphological development of *U. linza* axenic plantlets (treated with antibiotics) for 28 days.  
95 However, no single bacterium was able to completely restore normal morphology to axenic *U.*  
96 *linza*, in contrast to a recent observation in *U. mutabilis* applying bacteria isolated from *U. rigida*  
97 (Grueneberg *et al.*, 2016). Grueneberg *et al.* (2016) also demonstrated that *Ulva* can benefit from  
98 bacterial sources other than its own epiphytes, as diffusible waterborne morphogens can also  
99 affect *Ulva* development. This raises the question of specificity of the morphogen-producing  
100 bacteria.

101 To study microbial-algal interactions in the **laboratory**, strictly sterile (axenic) cultures of  
102 macroalgae pave the way for comparative research. Unlike other seaweeds, *Ulva* can be stably  
103 cultivated under laboratory conditions starting with axenic germ cells purified *via* their  
104 phototactic movement towards light, without applying antibiotics (Spoerner *et al.*, 2012;  
105 Wichard, 2015; Vesty *et al.*, 2015; Weiss *et al.*, 2017). The emerging model species *Ulva*  
106 *mutabilis* is routinely cultured in the **laboratory** with two bacteria, *Roseovarius* sp. strain MS2  
107 (GenBank EU359909) and *Maribacter* sp. strain MS6 (GenBank EU359911), which confer  
108 proper morphogenesis. The *U. mutabilis* used in laboratory experiments is a fast growing and  
109 naturally occurring developmental mutant ‘slender’ of *U. mutabilis* was used (Alsufyani *et al.*,  
110 2017). It shows only traces of the sea lettuce-like wildtype morphology and develops only  
111 primary rhizoids (Løvlie 1968; Wichard 2015). Axenic *U. mutabilis* cultures have an atypical  
112 “pincushion” morphotype, in which a lack of holdfast and exterior cell wall distortions are the  
113 main characteristics. Bacterially-derived substances govern rhizoid, cell wall and blade  
114 development (Spoerner *et al.*, 2012). Co-cultivation experiments using axenic gametes and MS2  
115 revealed that this bacterium promotes cell division and algal blade cell growth, analogous to  
116 cytokinin function in land plants. A similar experiment using MS6 showed that MS6 induces  
117 formation of a proper cell wall and a primary rhizoid, analogously to auxin in land plants  
118 (Spoerner *et al.*, 2012; Wichard, 2015). Overall these morphogenesis-inducing bacteria secreted  
119 a variety (i.e. MS6- and MS2-like factors) of still uncharacterised morphogenesis-inducing  
120 factors (= morphogens) into the culture medium of *U. mutabilis* (Spoerner *et al.*, 2012, Weiss *et*  
121 *al.*, 2017). The *U. mutabilis*-*Roseovarius*-*Maribacter* tripartite community established in the  
122 **laboratory** is an ideal model system with which to have controlled, repeatable conditions for  
123 further investigation of the interaction between a macroalga and its associated microbiome  
124 (Wichard *et al.*, 2015; Grueneberg *et al.*, 2016).

125 Very few studies have systematically addressed the still unanswered question of the species-  
126 specificity of epiphytic bacteria involved in the *Ulva*-bacterial interaction (Vesty *et al.*, 2015,  
127 Grueneberg *et al.*, 2016, Weiss *et al.*, 2017) and defined the microbiome, starting from purely  
128 axenic cultures, which could affect various morphogenetic traits (Spoerner *et al.*, 2012, Vesty *et*  
129 *al.*, 2015). This study reports on a cross-testing of potentially morphogenesis-inducing bacteria,  
130 isolated from various *Ulva* species, between the model system *U. mutabilis* and *U. intestinalis*.  
131 Phylogenetic analysis suggested a very close relationship between *U. intestinalis* and *U.*

132 *compressa* (Blomster *et al.*, 1998, Hayden *et al.*, 2003) and also, in spite of the variation in  
133 morphologies and life cycles, between *U. mutabilis* and *U. compressa* (Løvlie 1964; Tan *et al.*,  
134 1999; Wichard and Oertel, 2010). Phylogenetically well-characterized bacterial strains,  
135 originally isolated by Marshall *et al.*, (2006), were tested in a complementary bioassay, where  
136 test-strains replaced first one, and then the other, bacterium in the tripartite *U. mutabilis*-  
137 *Roseovarius-Maribacter* community (Spoerner *et al.*, 2012; Wichard 2015).

138

## 139 **Materials and Methods**

### 140 **Algal Samples**

141 Vegetative and fertile *U. intestinalis* blades were collected three times between March 2015 and  
142 April 2016 from Llantwit Major beach, South Wales, UK (51°40' N; 3°48' W). The sampling  
143 site was composed predominantly of *Ulva* species of a uniform morphology, mixed with brown  
144 algae in places. Excess water and epiphytic species were removed at the site by blotting the  
145 sample's surface before storage on ice for transport back to the laboratory. This species cannot  
146 be reliably identified solely using morphological characteristics, and thus plastid-encoded *rbcL*  
147 (large unit ribulose biphosphate carboxylase) and *tufA* (plastid elongation factor) markers were  
148 used for identification (see below). Haploid gametophytes from the fast-growing tubular mutant  
149 of *U. mutabilis* named *slender* (sl-G(mt+)) (Føyn, 1959, Løvlie, 1964) were used for all cross-  
150 testing and comparative investigations with *U. intestinalis*.

151

### 152 **Genomic DNA extraction from *Ulva*, amplification and sequence analysis of *rbcL* and *tufA*** 153 **genes.**

154 Genomic DNA was extracted from 25 mg seaweed samples using an ISOLATE II Genomic  
155 DNA Kit (Bioline, London, UK) according to the manufacturer's recommendations. DNA  
156 fragments of the *rbcL* and *tufA* genes were amplified by PCR using 30 ng DNA and 1 µl  
157 VELOCITY DNA Polymerase (2 units / µl) (Bioline Ltd, UK) in a final volume of 50 µl per  
158 reaction according to the manufacturer's protocol. Two primer pairs were used for *rbcL* marker:  
159 (i) Forward - *rbcL*Start 5'-ATGGCTCCAAAACTGAAAC-3', Reverse - 750 5'-  
160 GCTGTTGCATTTAAGTAATG-3' and (ii) Forward - F650 5'-  
161 GAAAACGTAAACTCACAACC-3', Reverse - *rbcL*End 5'-TTCTTTCCAAACTTCACA-3'.

162 The primers tested for *tufA* marker were *tufA* F 5'-GGNGCNGCNCAAATGGAYGG-3', *tufA* R  
163 5'-CCTTCNCGAATMGCRAAWCGC-3' (Fama *et al.* 2002).

164 The PCR conditions were as follows: *rbcL*- an initial denaturation step at 94°C for 2 min, 29  
165 cycles of 94°C for 45 s, 55°C annealing for 45 s and 90°C extension for 45 s. The cycles were  
166 succeeded by a final elongation step at 72°C for 7 min; *tufA*- an initial 4 min denaturation at 94  
167 °C, 38 cycles of 94°C for 1 min, 45°C annealing for 30 s, 72°C extension for 1 min, followed by  
168 72°C final extension for 7 min (Saunders *et al.*, 2010). PCR products were cleaned using the  
169 Thermo Fisher Scientific GeneJET™ PCR Purification Kit and sequenced on a capillary  
170 sequencer (ABI 3730, Applied Biosystems, USA) at the Functional Genomics Laboratory of the  
171 University of Birmingham.

172 The two primer pairs amplified two PCR products from the *rbcL* gene, 1-750 and 650-1430 (the  
173 3' end) that overlapped, meaning a sequence for almost the entire gene could be obtained by  
174 sequencing and aligning the PCR products. PCR products were fully sequenced from both ends  
175 using the primers used to amplify them. The resulting sequences were aligned manually (there  
176 were no mismatches in the double reads for each PCR product) using the overlapping central  
177 100bp (650-750) to generate a consensus *rbcL* sequence for submitting to GenBank (accession  
178 numbers MF038885). A single PCR product was generated for *tufA*, which was sequenced from  
179 both ends. Alignment of the forward and reverse *tufA* sequences demonstrated that they were  
180 identical, and a final consensus sequence of 772 bp was submitted to Genbank (MF162336).

181 The consensus sequences enabled the *Ulva* sample to be identified to species level by comparing  
182 the acquired sequence data with already-available sequence data in GenBank by using a Basic  
183 Local Alignment Search Tool (BLASTN; Johnson *et al.* 2008). Our sequences each had 100%  
184 match to only *Ulva intestinalis* samples.

### 185 **Cultivation Conditions**

186 The mutant slender (sl-G(mt+)) strain of *U. mutabilis* was propagated from unmated gametes  
187 derived from lab-grown parthenogenetic gametophytes. *U. intestinalis* was propagated from  
188 gametes derived from beach-collected gametophytes. All gametophytes were cultured in sterile  
189 culture flasks with gas-permeable screw caps (Nunc Int., Denmark) containing 100 mL *Ulva*  
190 Culture Medium (UCM; Stratmann *et. al.*, 1996) under the standard growth conditions including



191 a 17:7 h light/dark regime at 18 °C with an illumination of about 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$   
192 provided by 50 % GroLux, 50% day-light fluorescent tubes (Stratmann *et al.*, 1996).

193

#### 194 **Axenic Cultures**

195 Briefly, for preparation of axenic cultures, gametophytes of *U. mutabilis* and *U. intestinalis* were  
196 artificially induced to form gametangia by removal of at least two sporulation inhibitors  
197 (Stratmann *et al.*, 1996; Vesty *et al.*, 2015). Afterwards, on the third morning in daylight,  
198 gametes were released from the gametangia by an additional medium change and removing the  
199 swarming inhibitor (SWI) (Wichard and Oertel, 2010). Freshly-released gametes were purified  
200 from their accompanying bacteria by taking advantage of the gametes' fast movement towards  
201 light through a narrow horizontal capillary under strictly sterile conditions in a laminar flow  
202 hood. This method was repeated at least three times to obtain axenic gametes. As final step,  
203 bacterial contamination was checked by plating a drop of the 'gamete solution' on Marine Agar  
204 plates (Roth, Karlsruhe, Germany, supplemented with 1 % agar) and by PCR amplifications of  
205 the 16S rDNA (Spoerner *et al.*, 2012; Wichard, 2015).

206

#### 207 **Bacterial Strains**

208 By using axenic gametes in a standardized bioassay, it is possible to determine which microbes  
209 induce the algal morphogenesis through morphogenetically active substances (morphogens)  
210 (Grueneberg *et al.*, 2016). A large collection of *Ulva*-associated bacteria was available, isolated  
211 by the Callow **laboratory** (Marshall *et al.*, 2006; Marshall, 2004). These bacterial strains isolated  
212 from multiple *Ulva* species (including *U. linza*, *U. lactuca*, *U. compressa* and *Enteromorpha* sp.)  
213 have been maintained at -80 °C in glycerol as source cultures since collection: not all have been  
214 previously assigned a genus (Marshall *et al.*, 2006; Marshall 2004; J. Callow unpublished; Table  
215 1). UL19, EC19, UL16, EC34, E1, UL2 were selected, which induced a wide range of degrees of  
216 growth of axenic *Ulva* plantlets (based on (Marshall *et al.*, 2006) or our preliminary tests; Tables  
217 1 and 2).

218

#### 219 **Phylogenetic Characterization of Bacteria**

220 Ten  $\mu\text{L}$  of each of bacterial isolate was cultivated in 10 mL Marine Broth (MB; Roth, Karlsruhe,  
221 Germany) and then directly streaked onto Marine Agar plates to obtain single colonies. The  
222 plates were incubated at 20 °C for 5 days, then distinct colonies were picked off and transferred  
223 with a sterile loop into new bottles containing 10 mL MB. Bacterial DNA was extracted  
224 according manufacturer's instructions using a DNeasy Blood and Tissue kit (Qiagen, Hilden,  
225 Germany). To identify, or re-classify, the identity of the 6 bacterial strains using up-to-date  
226 classifications, partial 16S rDNA sequences (approx. 1500 bp) were amplified from these strains  
227 using the primer pair 27f (GGG TTT GAT CCT GGC TCA G) and 1390r (ACG GGC GGT  
228 GTG TRC AA) (Burggraf *et al.*, 1992; Olsen *et al.*, 1986). The reaction master mix contained  
229 2.5  $\mu\text{L}$  of PCR buffer 10% (100 mmol  $\text{L}^{-1}$  Tris/HCl pH 8.3, 500 mmol  $\text{L}^{-1}$  KCl, 15 mmol  $\text{L}^{-1}$   
230  $\text{MgCl}_2$ ), 1.25  $\mu\text{L}$  of BSA (20 mg/ml), 1 $\mu\text{L}$  each of forward and reverse primer (20 mM), 0.5  $\mu\text{L}$   
231 dNTPs 100 mM (dATP, dCTP, dGTP, dTTP), 0.15  $\mu\text{L}$  Taq polymerase (5 units /  $\mu\text{l}$ ), and ~100  
232 ng of template DNA. The PCR protocol included a 5-min initial denaturation at 95°C, followed  
233 by 31 cycles at 95°C for 30 s, 58 °C for 30 s, 72 °C for 90 s, finally 1 cycle of 7 min at 72 °C and  
234 storage at 4 °C. PCR products then were subjected to forward primer sequencing using the chain  
235 termination method (GATC, Göttingen, Germany). The closest homologous sequences in the  
236 GenBank database were recorded in Table 2. Two isolates belonged to the phylum  
237 *Proteobacteria* (*Alphaproteobacteria* class), two to the phylum *Actinobacteria*, one to the  
238 phylum *Bacteroidetes* and one belonged to the phylum *Firmicutes* (Table 2).

239

#### 240 **Bioassay-Guided Testing of Algal Morphogenesis Inducing Bacteria Associated with *Ulva***

241 To survey the activity of potentially morphogenesis-inducing bacteria, the '*Ulva* bioassay array'  
242 based on a multiwell plate format was used (Spoerner *et al.*, 2012; Grueneberg *et al.*, 2016).

243 To avoid any bias and thus minimize variation between several experimental set ups that would  
244 make the results ambiguous, positive and negative controls were run on identically prepared 96-  
245 well plates at the same time. As positive controls, *U. mutabilis* axenic gametes were incubated  
246 with the well-characterised *Roseovarius* sp. strain MS2 alone, *Maribacter* sp. strain MS6 alone  
247 and MS2+MS6 (triplicates of each) (as in (Spoerner *et al.*, 2012; the taxonomy of MS2 and MS6  
248 were recently reclassified by Grueneberg *et al.*, (2016)).

249 The same treatments were also carried out with axenic gametes of *U. intestinalis*. As a negative  
250 (axenic) control, 12 wells in one row were left without any bacterial inoculation in each plate.

251 For further comparison and evaluation, *U. intestinalis* was grown in flasks with the normal  
252 complement of *U. intestinalis*-associated bacteria by using non-purified gametes. Three  
253 biological replicates were conducted in parallel for each experiment.

254 The stock solution of freshly prepared axenic gametes was diluted with UCM to obtain the  
255 optimum concentration of gametes (about 300 gametes / mL). The density of gametes in the  
256 axenic stock solution was measured by flow cytometry (BD Accuri® C6) by comparing gamete  
257 samples to standards provided by the manufacturer (BD Biosciences, New Jersey, USA). The  
258 gamete solution was distributed in 96-well multiwell plates, 100 µL in each well. After  
259 incubation of plates overnight at room temperature in darkness, gametes homogenously settled  
260 down to the bottom of plates.

261 To observe the morphogenetic effects of *Ulva*-associated bacteria, *U. intestinalis* and *U.*  
262 *mutabilis* (*slender*, gametophyte, mt[+]) axenic gametes were inoculated with the bacteria  
263 isolated from three different *Ulva* species and *U. mutabilis*, individually and in combinations  
264 (triplicates of each) (Figs. 1, 2 and 3) as recently established by Weiss *et al.*, (2017). Bacterial  
265 strains were grown in marine broth for 3-7 days depending on the strain. The OD of the bacteria  
266 was measured and each strain was diluted in UCM to an OD of 1.0 and then serially diluted in  
267 additional UCM to a concentration of  $10^{-4}$  cells ml<sup>-1</sup>. Ten µl of this “stock” solution was then  
268 added to 100 µl of UCM containing *Ulva* gametes in a multiwell plate, giving a final  
269 concentration of  $10^{-5}$  bacterial cells ml<sup>-1</sup>. **The same “pattern” of bacterial strains was used on**  
270 **each plate, with plates growing under homogeneous light conditions and controlled temperature**  
271 **(Stratmann et al., 1996). Up to 5 technical repeats were carried out for each of 3 biological**  
272 **repeats – in each biological repeat, each plate was in a different position in the growth chamber,**  
273 **reducing the risk of “pseudo-replication”.** To avoid any contamination, plates were covered with  
274 gas permeable sealing film (Breathe-Easy, Diversified Biotech, MA, USA) and transferred to  
275 growth chamber under standard conditions (Wichard and Oertel, 2010). Over the next three  
276 weeks, plantlets were observed under the inverted microscope (DM IL LED, Leica, Wetzlar,  
277 Germany). The qualitative features considered under microscopic observation included the  
278 presence of unusual cell wall protrusions (‘bubble-like’ structures), thallus length, and  
279 differentiated rhizoid cells (Spoerner *et al.*, 2012). Quantification of the average blade cell  
280 number and the percentage of thalli with entirely normal cell walls were carried out. Cell  
281 numbers were compared using one-way Analysis of Variance (ANOVA), with a Dunn's multiple

282 comparison posteriori test using SigmaPlot 13 software (Systat Software, San Jose, CA).  
283 Comparison of the activities of MS2 and MS2-like bacteria between *U. mutabilis* and *U.*  
284 *intestinalis* were compared using two-way ANOVA followed by a Dunn's multiple comparison  
285 test using SigmaPlot software.

## 286 **Results**

### 287 **Bioassay-guided classification of the bacteria-induced morphogenesis of *Ulva mutabilis***

288 As demonstrated by Spoerner *et al.*, (2012), axenic *U. mutabilis* plants develop a characteristic  
289 morphology with a lack of holdfast and distortions of the exterior cell wall (Fig. 1). The effect of  
290 six individual bacterial species isolated from *Ulva* species were assessed for their ability to  
291 “rescue” the morphology of axenic *U. mutabilis* gametes back towards the complete non-axenic  
292 state (Fig. 1). A range of different morphotypes were stimulated by the individual bacterial  
293 strains, but none of them could solely elicit complete algal morphogenesis and normal  
294 development of *U. mutabilis* (Fig. 1)

295 Various *Ulva* bacterial isolates were able to promote marked morphological changes in *U.*  
296 *mutabilis*. Three out of these four isolates, *Paracoccus* sp., strains E34 and UL2, as well as  
297 *Cellulophaga lytica* UL16 caused cell divisions, like the reference strain *Roseovarius* sp. MS2  
298 (Fig. 1). As previously observed, the release of the MS2-like factor was not genus-dependent.  
299 Although in previous studies the MS2-like factor was frequently assigned to genera from the  
300 *Alphaproteobacteria*, we now show that the specific morphogenetic activity of blade induction  
301 can also be carried out by *Cellulophaga* sp. (Fig. 1; Table 1).

302 However, as the MS2-like factor does not drive normal cell wall development and protrusions  
303 remained visible (Fig. 1), further bacteria are necessary to complement the functional traits and  
304 to complete *Ulva*'s morphogenesis. We show that the *Actinobacterium Microbacterium* sp. EC19  
305 possesses this activity and can induce both cell differentiation and cell wall formation, but failed  
306 to induce a proper blade, which is analogous to the activity of the reference strain MS6 (Fig. 1).  
307 The two other tested bacteria *Microbacterium* sp. UL19, and *Planococcus* sp. E1, had no distinct  
308 effect on the growth and morphology of *U. mutabilis* and at the end of the experiment, algae  
309 cultured with these bacteria resembled axenic controls (Fig. 1). In addition, the strain E1 seems  
310 to negatively interfere with MS6, as the typical morphogenetic activities of MS6 are not visible  
311 in the presence of E1 (Figs 1 and 2). Overall, this shows that the morphogenetic activity of

312 bacteria towards *U. mutabilis* is bacterial strain-specific rather than correlating with bacterial  
313 genus.

314

### 315 **Bioassay-guided classification of the bacteria-induced morphogenesis of *Ulva intestinalis***

316 To address the question of how *Ulva* species-specific the morphogenetic activities of bacteria  
317 are, axenic cultures of *U. intestinalis* were prepared through application of the methods originally  
318 developed for *U. mutabilis*. In the absence of epiphytic bacteria, *U. intestinalis* plantlets reverted  
319 to an undifferentiated callus of cells (Fig. 2, controls), similar to axenic plantlets of *U. mutabilis*  
320 (Spoerner *et al.*, 2012, Vesty *et al.*, 2015) with unusual colourless protrusions from the exterior  
321 cell wall instead of the normal tubular morphology (Fig. 2, controls). As observed for *U.*  
322 *mutabilis*, the mode of action of *Paracoccus* sp. E34, *Cellulophaga* sp. UL16 and *Paracoccus* sp.  
323 UL2 on *U. intestinalis* plantlets was indistinguishable from the activities of the control reference  
324 strain MS2 (compare Fig. 1 and 2). The same was true for the respective activity of  
325 *Microbacterium* sp. EC19. Under the influence of EC19 axenic gametes of the “slender” mutant  
326 develop into minute short rows of degenerated blade cells with normal cell walls and rhizoid  
327 formation. EC19 thus revealed similarity to the activity of the MS6-like factor with *U.*  
328 *intestinalis* in addition to its activity with *U. mutabilis* (Fig. 2, compare with the MS6-control).  
329 The strong effect on rhizoid formation was prominent, forming multiple secondary rhizoids (Fig.  
330 2).

331

### 332 **Semi-quantification of the morphogenesis inducing activity of bacteria**

333 For further evaluation, a more detailed analysis was conducted. The number of cells produced by  
334 developing *Ulva* plantlets (Fig. 3A, B) and the degree of formation of cell wall protrusions as a  
335 result of a lack of MS6-morphogens was determined (Fig. 3C, D). Upon the inoculation of  
336 axenic gametes of *U. mutabilis* with the strains E34, UL16 or UL2 the average cell numbers  
337 increased four-fold (Fig. 3A;  $p < 0.05$ ) within two weeks: these strains were therefore as active  
338 as the reference strain MS2. There was no significant difference between the activity of MS2 and  
339 the MS2-like bacteria E34, UL2 and UL16 on *U. mutabilis*: all bacteria can rescue the cell  
340 division to the same degree (Fig. 3). However, two-way ANOVA revealed that the  
341 morphogenetic-activity of the bacteria E34, UL16 and UL2 was significantly lower on *U.*  
342 *intestinalis* (Fig. 3B;  $p < 0.05$ ) than on *U. mutabilis* (Fig. 3A;  $p < 0.05$ ) within the two-week

343 bioassay. Overall, we conclude that differences in growth of both algae are due to slower growth  
344 rates of *U. intestinalis* compared to *U. mutabilis* rather than the mode of action of the factors  
345 released by the respective bacteria.

346

#### 347 **A new tripartite system established with *U. intestinalis* and *U. mutabilis***

348 The applied strains have been tested in previous studies with *U. linza* and bacterial activities  
349 were classified according morphological scores by Marshall *et al.*, (2006) (Table 1), but different  
350 functional traits for growth and morphogenesis were not determined at that time. Therefore, in  
351 our study, bacterial strains were selected according to their two main functional traits (Figs 1 and  
352 2) in order to define new tripartite communities with *U. mutabilis* (Fig. 4) or *U. intestinalis* (Fig.  
353 5). Importantly, there was no species-specificity between *U. intestinalis* and *U. mutabilis*,  
354 because a range of bacteria can perform their eco-physiological functions similarly in both  
355 species (Figs 1 and 2).

356 The morphogenesis of *U. intestinalis* and *U. mutabilis* axenic germlings completely recovered in  
357 co-cultivation experiments with *Microbacterium* sp. EC19, the only selected strain that could  
358 phenocopy the *Maribacter* sp. MS6, and in combination with any one of E34, UL16 or UL2,  
359 which phenocopy the *Roseovarius* sp. MS2 (Figs 4 and 5). Upon inoculations, bacteria grew and  
360 formed a cluster around the rhizoid of *U. intestinalis* (Figure 6A) resembling the tripartite *U.*  
361 *mutabilis*-*Roseovarius*-*Maribacter* system (Spoerner *et al.*, 2012). It is not clear whether a single,  
362 or both, bacterial species are present at the rhizoid or how they achieve this, as only some species  
363 of *Microbacterium* sp. EC19 and *Paracoccus* sp. E34 are motile (Kelly *et al.*, 2006). Starting  
364 with this biofilm, *U. intestinalis* continues growing in the presence of any of the specifically  
365 designed microbiomes (Fig. 5G-I). In summary, a newly standardized *U. intestinalis* tripartite  
366 system has been established with various pairs of bacterial symbionts isolated from multiple  
367 *Ulva* species (Fig. 6).

368

369

## 370 **Discussion**

371 This study, starting with axenic cultures, has shown that phylogenetically distinct bacteria  
372 isolated from *Ulva* species other than *U. mutabilis* possess morphogenetic activity and can be  
373 used in combination to set up a tripartite system in an established model and phenocopy the  
374 reference strains MS2 and MS6. We also show that that the economically important *U.*  
375 *intestinalis* can function similarly in a tripartite system. We have defined new “minimal”  
376 microbiomes that promote growth, development and morphogenesis in *U. mutabilis* and *U.*  
377 *intestinalis*. The morphogenetic activity of all positively tested bacterial strains was comparable  
378 with the activity found in sterile-filtered natural water samples collected from the lagoon Ria  
379 Formosa (Portugal) using the same standardized bioassay (Grueneberg *et al.*, 2016).

380 This is the first report demonstrating the activity of an MS2-like factor within the phylum  
381 *Bacteroidetes*. Although experiments with boiling extracts of the *Maribacter* sp. MS6 revealed  
382 that this strain produces an MS2-like factor as well, the morphogenetic compound is not released  
383 into the environment (Spoerner *et al.* 2012). In any case, it should be taken into account that  
384 different compounds could show similar eco-physiological activities on *Ulva*'s morphogenesis.  
385 Our data contrasts with Grueneberg *et al.*, (2016), who reported two isolates, *Algoriphagus* sp.  
386 and *Polaribacter* sp. that could each singly rescue complete morphology in *U. mutabilis*. This  
387 experiment reveals again that strains of the same genus, UL19 and EC19, can harbour different  
388 functional traits.

389 Until now, only very few *Actinobacteria* have been tested on *Ulva* species for their effect on  
390 algal morphogenesis (Marshall *et al.* 2006) and *Microbacterium* sp. EC19 is the first  
391 representative of this phylum with a defined activity to *U. mutabilis* and *U. intestinalis*.

392 Interestingly the phylum *Actinobacteria* was also one of the major beneficial bacterial phyla  
393 detected on *Gracilaria vermiculophylla* from the North Sea (Lachnit *et al.* 2011) and associated  
394 with *Laminaria* populations (Wiese *et al.*, 2009; Salaün *et al.*, 2010).

395

## 396 **Host specificity of epiphytic bacteria on *Ulva* species, or lottery theory?**

397 This study tested whether a consistent core community is necessary to drive complete  
398 morphogenesis of *Ulva* species or whether a range of bacterial isolates can phenocopy the algal  
399 phenotypes induced by the strains MS2 (*Roseovarius*) and MS6 (*Maribacter*).

400 Large-scale 16S rRNA gene sequencing of the bacterial populations present on various  
401 individual of *U. australis* demonstrated that a consistent core microbiota could not be detected,  
402 and a large number of bacterial individuals are able to colonize the algal surfaces (Burke et al.,  
403 2011a,b). The temporal and spatial comparisons carried out by Tujula et al., (2010) have  
404 revealed that the microbiota on *U. australis* varies considerably among the individuals collected  
405 from both the same, and three different, tidal pools and also over different seasons. Despite these  
406 considerable shifts, it also has been demonstrated that a set of bacterial epiphytes belonging to  
407 *Alphaproteobacteria* and *Bacteroidetes* remained stable over space and time, implying their  
408 possible significant role in function of this bacterial community (Tujula et al., 2010). However,  
409 bacteria belonging to the less-abundant phylum *Actinobacteria* on *Ulva*'s surface (Friedrich  
410 2012), can harbour strong (morphogenetic) effects on algal growth as demonstrated in our study.  
411 Bioassays testing bacteria-induced morphogenesis, starting with axenic cultures, provide a  
412 unique approach to assess the specificity of bacterial functional traits within bacteria-macroalga-  
413 interactions. Some evidence suggested that the activities of the strain MS6, promoting rhizoid  
414 growth and normal cell wall development, were rare, in contrast to the activity of strain MS2,  
415 which promotes growth and blade development. Therefore, the MS6-like factor was considered  
416 to be a genus-specific functional trait, also due to the fact that those marine bacteria are hard-to-  
417 culture (Wichard 2015, Grueneberg et al 2016). With the findings of the current study, we show  
418 for the first time that both functional traits can be delivered by more than one bacterial phylum.  
419 The tripartite community of *Ulva* and bacteria can be established as long as certain bacteria  
420 release compounds with cytokinin-like activity, whereas others provide an auxin-like activity  
421 (Fig. 6). Overall, our data support the competitive lottery hypothesis (Sale 1976, Burke et al  
422 2011b), which implies that waterborne morphogenetic compounds are provided by various  
423 bacteria within a specific niche (algal surface). This seems to be a random process, which is  
424 based on the presence of functional genes and their functional characteristics rather than on a  
425 requirement for bacteria to belong to particular taxonomic groups. Our study shows that in the  
426 **laboratory**, two species of green algae can use combinations of compounds derived from multiple  
427 species of bacteria to drive their correct morphogenesis, and we hypothesise that similar  
428 situations may arise in their natural environment, where algae are exposed to multiple bacteria  
429 and waterborne compounds.

430



431 **Conclusions**

432 Designed microbiomes were used to test the algal morphogenesis-inducing traits of bacteria in  
433 both the standard test strain *U. mutabilis* and a new algal species, *U. intestinalis*. **By adding**  
434 **different bacteria singly or in pairs to *Ulva* gametes, our bioassays revealed that: (i) more than**  
435 **one *Ulva* species (both *U. mutabilis* and *U. intestinalis*) can respond to the same range of**  
436 **bacteria that affect algal growth, development and morphology *via* microbial morphogens; (ii)**  
437 **there is specificity in the bacterial signals regulating algal development, e.g. with some bacteria**  
438 **inducing rhizoid formation; (iii) the functions of bacteria (i.e. promoting cell elongation and**  
439 **division versus cell differentiation/cell wall formation) cannot be assigned to a specific genus**  
440 **taxonomic group.** This study supports Grueneberg *et al.*, (2016) who showed that the presence of  
441 specific (epiphytic) bacteria does not directly matter as long as *U. mutabilis* perceives sufficient  
442 diffusible morphogenetic compounds even from bacteria in the vicinity of other *Ulva* species  
443 within a shared habitat.

444 Establishing an additional standardized tripartite community (model system) with more than one  
445 species of *Ulva* presents an ideal possibility for elucidating the complexity of algal-bacterial  
446 partnership. The combined use of the tripartite communities will help to increase understanding  
447 of algal growth and development, to shed light on the underlying mechanisms involved in the  
448 cross-kingdom cross-talk of algae and bacteria. As *U. intestinalis* is a widespread alga with  
449 biofouling properties, our research presents a new way of understanding and controlling the life  
450 cycle of an economically important alga.

451

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460

461 **Conflict of interest.** None declared.

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467 **Legends**

468

469 **Figure 1.** Morphogenesis assessment of *U. mutabilis* using the “*Ulva* bioassay array”. The  
470 multiwell-based testing system of morphogenetic activity using axenic gametes of *Ulva*  
471 *mutabilis* allows the fast determination of the different morphotypes induced by bacteria isolated  
472 from various *Ulva* species, singly and in pairwise combination with the bacteria *Roseovarius* sp.  
473 MS2 and *Maribacter* sp. MS6. Representative morphotypes are categorized by a color code:  
474 **Yellow circle (axenic morphotype):** Callus-like cultures with typical colorless cell wall  
475 protrusions. **Magenta circle (morphotype induced by the MS2-like factor):** Germlings with  
476 normal cell division towards one direction but still covered by protrusions and differentiated  
477 rhizoid cells are missing. **Red circle (morphotype induced by the MS6-like factor):** Plantlets  
478 show a proper cell wall and rhizoid formation but the blade does not develop. **Green circle**  
479 **(completely recovered morphotype):** Characteristic usual morphotype with normal blade and  
480 rhizoid formation. Propagules are three-weeks old. Controls are shown in the bottom row.  
481 Arrows indicate the typical colourless protrusions from the exterior cell walls of axenic cultures.  
482 Scale bars = 100  $\mu$ m.

483

484 **Figure 2.** Morphogenesis assessment of *U. intestinalis* using the “*Ulva* bioassay array”.  
485 Different morphotypes of *Ulva intestinalis* induced by bacteria isolated from various *Ulva*  
486 species singly and in pairwise combination with the bacteria *Roseovarius* sp. MS2 and  
487 *Maribacter* sp. MS6. Arrows indicate the typical colourless protrusions from the exterior cell  
488 walls of axenic cultures. Representative morphotypes are categorized by the same colour code as  
489 described in Figure 1. Propagules are three weeks old. Controls are shown in the bottom row.  
490 There was no significant differences in growth and morphology between propagules grown in  
491 the presence of the strains MS2 and MS6 compared to those grown in the presence of the natural  
492 microbiome (“Natural”). Scale bars = 100  $\mu$ m.

493

494 **Figure 3.** Semi-quantitative data of bacteria-induced growth and morphogenesis derived from  
495 the “*Ulva* bioassay array” with axenic *Ulva mutabilis* (A, C) and *Ulva intestinalis* (B, D)  
496 gametophytes. (A) and (B): To estimate the activity of the MS2-like factor, the total cell numbers  
497 in thalli of *Ulva mutabilis* (A) and *Ulva intestinalis* (B) plantlets were counted 10 days after

498 inoculation with *Microbacterium* sp. EC19, *Microbacterium* sp. UL19, *Planococcus* sp. E1,  
499 *Paracoccus* sp. E34, *Cellulophaga* sp. UL16 or *Paracoccus* sp. UL2. Controls show the  
500 morphogenetic activity on gametes without bacteria, with the bacterial strain MS2, with the  
501 bacterial strain MS6 and with both MS2 and MS6 bacterial strains. (C) and (D): To determine  
502 the activity of the MS6-like factor, the proportion of thalli of *Ulva mutabilis* (C) and *Ulva*  
503 *intestinalis* (D) with normal cell wall development was evaluated as a percentage of total thalli  
504 10 days after inoculation with bacteria listed above. A one-way ANOVA was performed to  
505 reveal statistically significant differences, followed by a Dunn's multiple comparison test to  
506 determine which groups differ ( $P < 0.05$ ), indicated by the letters a and b. Error bars represent  
507 (A, B) confidence intervals ( $P = 0.95$ ;  $n > 30$  individual algae) or (C, D) standard deviations ( $n >$   
508 30 individual algae).

509

510 **Figure 4.** Establishment of a tripartite community of *Ulva mutabilis* with novel bacteria. Three-  
511 week old *U. mutabilis* gametophytes are shown inoculated with bacteria isolated from different  
512 *Ulva* species in pairwise combination. Axenic gametes of *U. mutabilis* were inoculated with (A)  
513 *Microbacterium* sp. EC19 only, and together with (B) *Microbacterium* sp. UL19, (C)  
514 *Planococcus* sp. E1, (D) *Paracoccus* sp. E34, (E) *Cellulophaga* sp. UL16 or (F) *Paracoccus* sp.  
515 UL2. (D-F) Due to the complementary functional traits of the bacteria, the tripartite community  
516 can completely recover the morphogenesis of *U. mutabilis*, whereas the bacterial isolates UL19  
517 and E1 do not contribute to the algal development. The bioassay system was scaled up using  
518 sterile culture flasks. Scale bars = 100  $\mu\text{m}$ .

519

520 **Figure 5.** Establishment of a tripartite community of *Ulva intestinalis*. Three-week old *U.*  
521 *intestinalis* gametophytes are shown inoculated with bacteria isolated from different *Ulva* species  
522 in pairwise combination. Axenic gametes of *U. mutabilis* were inoculated with (A)  
523 *Microbacterium* sp. EC19 only, and together with (B) *Microbacterium* sp. UL19, (C)  
524 *Planococcus* sp. E1, (D) *Paracoccus* sp. E34, (E) *Cellulophaga* sp. UL16 or (F) *Paracoccus* sp.  
525 UL2. (D-F) Due to the complementary functional traits of the bacteria, the tripartite community  
526 can completely recover the morphogenesis of *U. intestinalis*. (G-I) The thallus of *U. intestinalis*  
527 continues growing under these conditions and increases significantly in size within one more  
528 week. (A-F) Scale bars = 100  $\mu\text{m}$  and (G-I) scale bars = 1 cm.

529

530 **Figure 6.** Model systems for bacteria-macroalgae interactions. (A) Bacterial biofilm formation:  
531 Upon inoculation of *Ulva intestinalis* axenic gametes with *Microbacterium* sp. EC19 and  
532 *Paracoccus* sp. E34 for 5 days, bacteria concentrate around the rhizoid. Scale bars = 10  $\mu$ m. (B)  
533 Effects of a defined microbiome can be reliably tested using tripartite systems of *Ulva mutabilis*  
534 or *Ulva intestinalis* and multiple combinations of algal morphogenesis-inducing bacteria. Figure  
535 was adapted and changed from Grueneberg et al. (2016). Names of bacterial strains, which were  
536 tested in this study for the first time, are printed in black.

537

538

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