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# 1 Changing microbial activities during low salinity 2 acclimation in the brown alga *Ectocarpus subulatus*

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18

19 **Running title:** *Ectocarpus* microbiome impacts stress tolerance

20 **Keywords:** Low salinity acclimation, host-microbiome interaction, virome, metabolic

21 networks, meta-transcriptomics, metabolite profiling, holobiont, brown algae

22

## 23 Originality-Significance Statement

24 The importance of symbiotic microbes for the health and stress resistance of multicellular  
25 eukaryotes is widely acknowledged, but understanding the mechanisms underlying these  
26 interactions is challenging. They are especially difficult to separate in systems with one or  
27 more uncultivable components. We bridge the gap between fully controlled, cultivable model  
28 systems and purely environmental studies through the use of a multi-omics approach and  
29 metabolic models on experimentally modified "holobiont" systems. This allows us to generate  
30 two promising working hypotheses on the mechanisms by which uncultivated bacteria  
31 influence their brown algal host's fresh water tolerance.

32

## 33 Summary

34 *Ectocarpus subulatus* is one of the few brown algae found in river habitats. Its ability to tolerate  
35 freshwater is due, in part, to its uncultivated microbiome. We investigated this phenomenon by  
36 modifying the microbiome of laboratory-grown *E. subulatus* using mild antibiotic treatments,  
37 which affected its ability to grow in low salinity. The acclimation to low salinity of fresh water-  
38 tolerant and intolerant holobionts was then compared. Salinity had a significant impact on  
39 bacterial gene expression as well as the expression of algae- and bacteria-associated viruses in  
40 all holobionts, albeit in different ways for each holobiont. On the other hand, gene expression  
41 of the algal host and metabolite profiles were affected almost exclusively in the fresh water  
42 intolerant holobiont. We found no evidence of bacterial protein production that would directly  
43 improve algal stress tolerance. However, we identified vitamin K synthesis as one possible  
44 bacterial service missing specifically in the fresh water-intolerant holobiont in low salinity.

45 We also noticed an increase in bacterial transcriptomic activity and the induction of microbial  
46 genes involved in the biosynthesis of the autoinducer AI-1, a compound that regulates quorum  
47 sensing. This could have caused a shift in bacterial behavior in the intolerant holobiont,  
48 resulting in virulence or dysbiosis.

## 49 Introduction

50 Brown algae are multicellular members of the stramenopile lineage and frequently form  
51 the dominant vegetation in intertidal zones of temperate marine coastal ecosystems (Wahl *et*  
52 *al.*, 2015). They are important both as ecosystem engineers and increasingly being exploited as  
53 a food source (Food and Agriculture Organization of the United Nations, 2016), and for the  
54 production of alginate (McHugh, 2003) or other high-value compounds (Milledge *et al.*, 2016;  
55 Silva *et al.*, 2020). Like virtually all macroorganisms, brown algae have formed tight  
56 relationships with their associated microbiota, which may provide them with vitamins,  
57 phytohormones, protection against disease or fouling, etc. (Goecke *et al.*, 2010; Wahl *et al.*,  
58 2012; Egan and Gardiner, 2016).

59 *Ectocarpus* is a cosmopolitan genus of small filamentous brown algae that is easy to  
60 cultivate in the laboratory but closely related to large kelp-forming brown algal species, at the  
61 evolutionary level. The *Ectocarpus* sp. strain Ec32 has been established as one of the key model  
62 systems to study brown algal biology (Charrier *et al.*, 2008), and its genome was the first brown  
63 algal genome to be published (Cock *et al.*, 2010). *Ectocarpus* is furthermore a model to study  
64 algal bacterial interactions, with studies demonstrating, for instance, its reliance on bacteria-  
65 produced cytokinins, which serve as morphogens for the alga (Pedersén, 1968, 1973; Tapia *et*  
66 *al.*, 2016). More recently, metabolic complementarity has been successfully used in this system,  
67 highlighting potentially beneficial metabolic host-microbe interactions (Dittami *et al.*, 2014;  
68 Burgunter-Delamare *et al.*, 2020).

69           One species of *Ectocarpus*, *Ectocarpus subulatus*, is of particular interest, as it has been  
70 described in river habitats (West and Kraft, 1996; Dittami, Peters, *et al.*, 2020), yet its capacity  
71 to tolerate freshwater medium in the laboratory seems to be conditioned by the presence of a  
72 specific microbiota (Dittami *et al.*, 2016). We speculate that this dependence on bacteria may  
73 be related to (i) the direct production of compounds such as osmolytes or chaperones that  
74 enhance algal low salinity tolerance, (ii) general bacterial services that are required for algal  
75 growth regardless of the salinity, (iii) protective functions of some bacteria to prevent  
76 phenomena of dysbiosis, or (iv) a combination of these effects. However, despite extensive  
77 cultivation efforts (KleinJan *et al.*, 2017) followed by co-culture experiments (personal data),  
78 neither the bacteria responsible for this phenomenon nor the underlying bacterial functions have  
79 been identified so far, possibly because (part of) the bacteria responsible are found within the  
80 uncultivable part of the microbiome.

81           The role of *Ectocarpus*-associated bacteria in the fresh water response is investigated in  
82 this study using a novel approach. Rather than starting from a collection of cultured bacteria,  
83 we worked with algal holobionts that had been treated with various antibiotic combinations.  
84 These antibiotics were powerful enough to change the algal microbiome, but not completely  
85 eliminate it. Several of the treated algae were able to survive in freshwater. We then used a  
86 combination of metagenomics, meta-transcriptomics, and metabolomics to investigate three  
87 algal holobionts, each with its own microbiome and response to fresh water. It allowed us to  
88 develop testable hypotheses about how these holobionts respond to low salinity and, in  
89 particular, how changes in bacterial composition and activity correlate with the alga's  
90 acclimation capacity.

## 91 Material and methods

### 92 Preparation of algal holobionts with modified microbiomes

93 Starter cultures of *Ectocarpus subulatus* freshwater strain (EC371, accession CCAP  
94 1310/196, West & Kraft, 1996) were grown in 90 mm Petri dishes in natural seawater (NSW;  
95 salinity 35, collected in Roscoff 48°46'40"N, 3°56'15"W, 0.45 µm filtered, autoclaved at 120°C  
96 for 20 min), enriched with Provasoli nutrients (2 mg·L<sup>-1</sup> Na<sub>2</sub>EDTA, 2.24 mg·L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 240  
97 µg·L<sup>-1</sup> MnSO<sub>4</sub>·H<sub>2</sub>O, 44 µg·L<sup>-1</sup> ZnSO<sub>4</sub>·7 H<sub>2</sub>O, 10 µg·L<sup>-1</sup> CoSO<sub>4</sub>·7H<sub>2</sub>O, 0.7 mg·L<sup>-1</sup> FeEDTA,  
98 0.6 mg·L<sup>-1</sup> Na<sub>2</sub>EDTA, 4 mg·L<sup>-1</sup> Na<sub>2</sub> β-glycerol PO<sub>4</sub>·5H<sub>2</sub>O, 35 mg·L<sup>-1</sup> NaNO<sub>3</sub>, 0.875 µg·L<sup>-1</sup>  
99 vitamin B12, 40 µg·L<sup>-1</sup> thiamine, 4 µg·L<sup>-1</sup> biotin; Starr and Zeikus 1993). They were kept at  
100 13°C with a 12h dark-light cycle (photon flux density 20 µmol m<sup>-2</sup>·s<sup>-1</sup>). Starter cultures were  
101 then treated with different antibiotics, and their capacity to grow in diluted natural seawater  
102 medium (5% NSW, 95% distilled water) was assessed (Supplementary Table S1). Three  
103 "holobionts" were selected for the final experiments (Table 1). Two of them (H1, H2) were  
104 based on short (3 days) treatments with antibiotics solutions and were capable of growing in  
105 diluted natural seawater medium. The last one (H3) was subjected to 5 weeks of antibiotic  
106 treatment on Petri dishes and was no longer fresh water-tolerant. After recovery of two weeks  
107 (H1, H2) to five months (H3) in antibiotic-free 100% NSW, cultures were transferred to 10 L  
108 culture flasks, grown for approximately two months to obtain sufficient biomass, and then split  
109 into ten replicate cultures in 2 L culture flasks (each replicate with identical microbial  
110 communities). To ensure that the holobionts differed in their microbial community  
111 composition, and before acclimation experiments, two replicates samples of each culture  
112 (technical replicates) were used for 16S rRNA gene metabarcoding. The experimental setup is  
113 shown in Figure 1.

## 114 16S rRNA gene metabarcoding

115 16S rRNA metabarcoding was carried out as previously described (KleinJan *et al.*,  
116 2017). Briefly, total DNA was isolated (NucleoSpin Plant II, Machery-Nagel; standard  
117 protocol) from snap-frozen tissue and purified with Clontech CHROMA SPINTM-  
118 1000+DEPC-H2O columns. The V3–V4 region of the 16S rRNA gene was amplified and  
119 sequenced with Illumina MiSeq technology by MWG Eurofins Biotech (Ebersberg, Germany)  
120 using their proprietary protocol and yielding 1,859,076 reads. After quality trimming using the  
121 FASTX Toolkit (quality threshold 25; minimum read length 200), data were analyzed with  
122 Mothur (V.1.38.0) according to the MiSeq Standard Operating Procedures (Kozich *et al.*,  
123 2013). Sequences were aligned to the non-redundant Silva SSU reference database version 123  
124 (Quast *et al.*, 2013), chimeric sequences removed using Uchime (Edgar *et al.*, 2011), clustered  
125 into operational taxonomic units (OTUs) at a 97% identity level, and classified taxonomically  
126 (Wang *et al.*, 2007).

## 127 Acclimation experiments

128 Acclimation experiments were carried out to elucidate differences in the response of the  
129 three holobionts to low salinity. Because natural seawater medium diluted to 5% NSW was  
130 lethal to the non-tolerant holobionts (H3) and based on preliminary screening experiments, we  
131 opted for a final concentration of 15% NSW and 85% distilled water enriched with Provasoli  
132 nutrients. At 15% NSW, growth in the non-tolerant holobiont (H3) was inhibited, but the  
133 condition was not lethal. Five replicate cultures of each holobiont (prepared as described above)  
134 were transferred from 100% NSW to 15% NSW, and the other five replicates were transferred  
135 to fresh 100% NSW as a control. After one-week, algal tissue was harvested using UV-sterilized  
136 coffee filters. Excess water was removed by drying with a clean paper towel, tissues snap-  
137 frozen in liquid nitrogen, and stored at -80 °C until further processing.

## 138 Metagenome and metatranscriptome sequencing

139           Approximately 50 mg (fresh weight) of algal tissue were ground in liquid nitrogen and  
140 sterilized sand using a pestle and mortar. Nucleic acids were extracted as previously described  
141 by Le Bail *et al.* (2008) using a CTAB-based lysis buffer and phenol-chloroform purification.  
142 RNA and DNA were separated after precipitation with LiCl (0.25V, 12M, overnight, -20°C);  
143 the RNA was resuspended in 300 µl RNase free and purified with 1 volume of phenol:  
144 chloroform (1:1, pH 4.3, 20 min., 4°C) and twice with one volume of chloroform. It was then  
145 precipitated (0.1V 3M sodium acetate, 3V 100% ethanol, 2h at -80°C), washed with ice-cold  
146 70% ethanol, and finally resuspended in 15 µl RNase free water. Ribosomal rRNA molecules  
147 were depleted from the RNA extracts using the RiboMinus™ Plant Kit for RNA-Seq  
148 (ThermoFisher Scientific, Waltham, MA, USA). This allowed to selectively remove abundant  
149 nuclear, mitochondrial, and chloroplast rRNAs of the algal host. Besides, 1 µl of the bacterial  
150 probe (RiboMinus™ Transcriptome Isolation Kit for Yeast and Bacteria, ThermoFisher  
151 Scientific) was added in the last five minutes of hybridization to remove bacterial ribosomal  
152 RNA. The RNA was concentrated (RiboMinus™ Concentration module, ThermoFisher  
153 Scientific) before checking the quality with a bioanalyzer (Agilent, Santa Clara, CA, USA).  
154 Library preparation (TruSeq Stranded mRNA Library Prep kit, Illumina) and sequencing (five  
155 lanes, 150bp read length, paired-end, Illumina HiSeq 3000, GeT PlaGe Genotoul, Auzeville,  
156 France) were carried out for four of the five replicates per condition. The fifth replicate was  
157 kept as a backup.

158           DNA was extracted from the supernatant of LiCl precipitation. It was precipitated with  
159 one volume of isopropanol, resuspended in 300 µL DNA-free water, purified once with one  
160 volume of phenol:chloroform:isoamyl alcohol (25:24:1; pH 8), and twice with one volume of  
161 chloroform. Finally, the DNA was precipitated (3 volumes of 100% EtOH + 0.1 volumes of  
162 3M sodium acetate) and resuspended in 50 µL of molecular biology grade water. DNA extracts

163 from all samples were pooled (same concentration of each sample), and the pooled DNA was  
164 purified using cesium chloride gradient centrifugation (Le Bail *et al.*, 2008). A library was  
165 constructed using the Illumina TruSeq DNA Nano kit and was sequenced on four lanes of  
166 Illumina HiSeq 3000 (150bp read length, paired-end, GeT PlaGe Genotoul platform). DNA and  
167 RNA sequencing data were deposited at the European Nucleotide Archive (ENA) under project  
168 accession PRJEB43393.

## 169 [Metagenome analyses](#)

170 Raw sequencing reads were quality-trimmed with Trimmomatic (version 0.36, minimal  
171 Phred score: 20, minimal read length: 36 nucleotides) and aligned to the *E. subulatus* Bft15b  
172 reference genome (Dittami, Corre, *et al.*, 2020) using STAR aligner version 2.6.0a (Dobin *et*  
173 *al.*, 2013) to remove algal reads. Non-aligning (*i.e.* bacterial) reads were then assembled using  
174 MetaSPAdes (Nurk *et al.*, 2017). The resulting contigs were filtered by length (>500 bp), and  
175 the remaining *Ectocarpus* contigs were removed with Taxoblast version 1.21 (Dittami and  
176 Corre, 2017). Metagenomic binning was carried out using Anvi'o version 4.0 according to the  
177 "Anvi'o User Tutorial for Metagenomic Workflow" (Eren *et al.*, 2015): Raw metagenome reads  
178 were mapped against the contigs database using BWA-MEM (version 0.7.15), and taxonomy  
179 was assigned to the contigs using Centrifuge (Kim *et al.*, 2016) and the NCBI nucleotide non-  
180 redundant database (nt\_2018\_3\_3). Finally, contigs were clustered according to GC content  
181 and coverage, and the Anvi'o interactive interface was used for manual curation of the bins. The  
182 quality of the metagenomes was assessed based on the abundance of single-copy core genes  
183 (SCGs; Campbell *et al.*, 2013). One bin containing essentially *Ectocarpus* reads that had been  
184 missed during the previous cleaning steps was manually removed at this stage. The remaining  
185 bins were bacterial and annotated with Prokka v1.13 (Seemann, 2014).

186 Metabolic networks were reconstructed using Pathway Tools version 20.5 (Karp *et al.*,  
187 2016) and the scripts included in the AuReMe pipeline (Aite *et al.*, 2018). This dataset served  
188 as a backbone for the analysis of bacterial gene expression from the metatranscriptomic data.

### 189 (Meta)transcriptome analyses

190 Ribosomal RNA reads that remained despite the RiboMinus treatment were removed *in*  
191 *silico* using SortMeRNA version 2.1. After quality trimming (Trimmomatic 0.36, minimal  
192 Phred score: 20, minimal read length: 36 nucleotides), reads were mapped first to the *E.*  
193 *subulatus* Bft15b reference genome for algal gene expression analysis and the remaining reads  
194 to the metagenomic bins generated as described above using STAR aligner version 2.6.0a. The  
195 ratio of bacterial to algal mRNA was compared across samples using a one-way ANOVA  
196 followed by Tukey's HSD test carried out using Past v4 (Hammer *et al.*, 2001). Then, both the  
197 algal transcriptome and the bacterial metatranscriptome were analyzed separately. In both  
198 cases, DESeq2 (Love *et al.*, 2014) was used for principal component analysis (PCA, rlog-  
199 transformed data) and for the detection of significantly differentially expressed genes (DEGs).  
200 However, for the bacterial metatranscriptome, read coverage was insufficient to carry out  
201 differential gene expression analysis on a bin per bin basis. Therefore, we summed up the  
202 bacterial expression data associated with the same metabolic reaction across the different  
203 metabolic networks. This overall transcription of a metabolic function within the entire  
204 microbiome was used to identify differentially expressed microbial reactions in the same way  
205 as the algal DEGs.

206 For both the algal transcriptome and the bacterial metabolic reactions, the following  
207 comparisons were carried out. First, each of the three holobionts (H1, H2, H3) was analyzed  
208 individually to determine genes and reactions that were differentially expressed in 15% NSW  
209 compared to 100% NSW. Then, the same conditions of the two freshwater-tolerant holobionts

210 were grouped (i.e. H1-100% and H2-100% were grouped as well as H1-15% and H2-15%), and  
211 these newly formed groups were compared to the holobiont that was not able to grow in  
212 freshwater (H3-100% and H3-15%). For this comparison, the statistical design considered both  
213 factors (holobiont and salinity) and the interaction term. All genes or reactions with an adjusted  
214 p-value <0.05 and a fold change in expression > 1.5 were considered significantly differentially  
215 expressed. Gene set enrichment analyses were performed for sets of differentially expressed  
216 genes using the Fisher's exact module within Blast2Go (Version 4.1.9; 2-tailed test; FDR <  
217 0.05; Götz *et al.* 2008). Metabolic reactions were associated with metabolic pathways according  
218 to the MetaCyc database v20.5, and pathways with >50% of differentially expressed metabolic  
219 reactions were further examined.

220 For the bacterial metatranscriptomes, in addition to differentially expressed metabolic  
221 reactions, we also determined the overall "transcriptomic activity" of each bin in each condition.  
222 To this means, read pair counts were summarized for all genes of the same bin and normalized  
223 by the total number of mapping read pairs per sample. The resulting matrix was used as input  
224 for hierarchical clustering (distance: correlation; method: average) with ClustVis (Metsalu and  
225 Vilo, 2015).

226 Finally, viral reads present in the metatranscriptomes were analyzed using the  
227 Metagenomic RAST (MG-RAST) pipeline (Meyer *et al.*, 2008). Sequencing reads were  
228 annotated using the best-hit annotation tool against the RefSeq database (Pruitt *et al.*, 2007).  
229 Taxonomic affiliations were generated at the family level if possible, and the BLAST  
230 parameters were set to the default settings of MG-RAST (15aa minimal alignment and 1E-05  
231 e-value threshold). The extracted number of reads for viruses was Hellinger-normalized and  
232 used as input for ANOVA and PCA analyses using STAMP v2.1.3 (Parks *et al.*, 2014).

233

## 234 Metabolite profiling by gas-chromatography

235 For metabolite profiling, 10 mg of freeze-dried ground tissue was lysed (TissueLyser,  
236 2\*30s, 30 Mhz, Qiagen, Hilden, Germany) and subsequently used for extraction of metabolites  
237 with 1 ml of 100% methanol (Dittami *et al.*, 2012). After vortexing, 5 µl of ribitol (4 mM in  
238 H<sub>2</sub>O, >99%, Sigma-Aldrich, Munich, Germany) were added to each sample as an internal  
239 standard before sonication (10 min, at room temperature; RT). After 15 minutes of  
240 centrifugation (30,000 g, 4 °C) the supernatant was recovered, evaporated under vacuum  
241 overnight, derivatized in 50 µl methoxymation solution (20 mg/ml in pyridine), and incubated  
242 at 60 °C for 1h and afterward at RT overnight. The samples were silylated for 1h at 40°C in 50  
243 µl MSTFA (1 ml + 40 µl retention index mix) and centrifuged (6 min, 2500 rpm) to pellet the  
244 precipitate (Alsufyani *et al.*, 2017). The supernatant was analyzed with a 6890N gas  
245 chromatograph, equipped with a 7683B autosampler (Agilent), a glass liner (Agilent, 4 × 6.3 ×  
246 78.5 mm), and a DB-5MS column (Agilent, 30 m × 0.25 mm × 0.25 µm), coupled to a  
247 Micromass GCT Premier™ (Waters®) mass spectrometer. The gas chromatograph was  
248 operated with helium as a mobile phase, split 10, and 250°C injector temperature. The initial  
249 oven temperature was 60°C ramping to 310°C at a rate of 15°C per min. The mass spectrometer  
250 was used with a source temperature at 300°C and dynamic range extension mode. The  
251 resolution was > 6,000 FWHM at *m/z* 501.97. After randomization, samples were measured  
252 twice to obtain two technical replicates. Solvent blanks were prepared and measured in parallel.

253

## 254 Analysis of metabolite data

255 Raw files were directly converted to the netCDF format using the DataBridge tool  
256 within the MassLynx software (Waters, version 4.1), and the chromatograms were then  
257 processed with the function metaMS.runGC (version 1.0) provided within

258 Workflow4Metabolomics (W4M) (Giacomini *et al.*, 2015). The metaMS package (Wehrens *et*  
259 *al.*, 2014) was used to identify chromatographic peaks with the standard functions provided by  
260 XCMS. Then, the CAMERA package was used to cluster masses with similar retention times  
261 (Kuhl *et al.*, 2012). These co-eluting masses or 'pseudospectra' were summarized into a final  
262 feature table in the MSP format, a format that can be used to search in spectral databases. A  
263 detailed list of settings can be found in [Supplementary Table S2](#). The resulting matrix of 689  
264 features (pseudospectra) was manually processed. To remove any contaminant signals from the  
265 matrix, each peak's maximum value among all blanks was multiplied by three and subtracted  
266 from the remaining samples. Variables with less than two samples with intensities above zero  
267 and redundant ions (isotopes) were removed. The filtered datasets were then re-imported into  
268 W4M for statistical analysis. Quality assessment of the data confirmed that there were no  
269 outliers and there was no signal drift. Data were normalized by dry weight followed by log<sub>10</sub>-  
270 scaling and a student *t*-test was used to detect metabolites that were significantly different  
271 (adjusted p-value < 0.05) in each holobiont during the shift from 100% NSW to 15% NSW,  
272 and between holobionts H1/H2 and holobiont H3 in the 100% NSW condition. Finally, the  
273 spectra of each significant feature were compared to the GOLM libraries (Hummel *et al.*, 2010)  
274 and an in-house library (Kuhlisch *et al.*, 2018) for annotation using NIST MS Search (version  
275 2.0). Features with a reverse match score (R)  $\geq 800$  were annotated, for  $700 \leq R < 800$  features  
276 were annotated but labeled with an additional "?", and for  $600 \leq R < 700$  they were labeled with  
277 "??". Clustering was carried out using ClustVis as described above for the metatranscriptome  
278 data.

## 279 Results

### 280 Sequencing data

281 Illumina sequencing resulted in a total number of 2.9 billion metagenomic reads and 3.2  
282 billion RNAseq reads (average of 2.7 million per sample). Roughly half of the metagenome  
283 reads mapped with the algal genome, and the other half was considered bacterial. For the  
284 RNAseq data, despite *in vitro* ribodepletion, on average, 81% of reads corresponded to  
285 ribosomal sequences, and the remaining reads mapped to the alga (both nuclear 2-11% and  
286 organellar reads 1-21%). Only 0.4 to 12% (3% on average) of the total reads did not map to the  
287 algae (see [Supplementary Table S3](#) for details) and were considered bacterial or viral. The ratio  
288 of bacterial to algal mRNA varied significantly according to treatment (one-way ANOVA,  $p <$   
289 0.001) and was highest in H3 in low salinity, which differed significantly from all other  
290 treatments according to a Tukey HSD test ( $p < 0.001$ , [Figure 2](#)).

### 291 The bacterial metagenome

292 Metagenome sequencing of pooled DNA of all samples was carried out to generate a  
293 reference for the analysis of bacterial gene expression. The assembly of metagenome reads not  
294 mapping to the algal genome resulted in a total of 145,058 contigs corresponding to 332 Mbp  
295 of sequence information. Anvi'o binning of these contigs yielded 73 bins ([Figure 3](#)), as well as  
296 one bin that was created artificially to regroup all contigs that did not fall into any other well-  
297 defined bin (19 Mbp of sequence data). Thirty-five metagenomic bins had a completeness  $\geq$   
298 90% (categorized as "full"). Four of those were  $\geq 10\%$  redundant (Bin 29: 18%, Bin 61: 18%,  
299 Bin 74: 16%, Bin 42: 21%). Thirty-eight bins were  $\leq 90\%$  complete and categorized as  
300 "partial". Most bins were taxonomically assigned to *Proteobacteria* (53), followed by  
301 *Bacteroidetes* (11) *Planctomycetes* (3), *Actinobacteria* (3), and unclassified bacteria (3).  
302 Among the *Proteobacteria*, *Alphaproteobacteria*, specifically *Rhizobiales* (12) and

303 *Rhodobacterales* (15), were the most abundant. *Bacteroidetes* were comprised of six  
304 *Flavobacteria* and one *Cytophaga*. A complete overview of the bacterial bins and assembly  
305 statistics can be found in [Supplementary Table S4](#). Each of these genomic bins was annotated,  
306 and metabolic networks were created. The merged metabolic network of all bacterial bins  
307 comprised 3,957 different metabolic reactions ([Supplementary Table S5](#)).

### 308 [Algal gene expression](#)

309 The PCA plots of algal gene expression show three groups ([Figure 4A](#)). The first group  
310 comprises the freshwater-tolerant holobionts H1 and H2 regardless of the salinity of their  
311 culture medium, and the other two groups correspond to the freshwater-intolerant holobiont in  
312 low (H3-15%) and full salinity (H3-100%), respectively. This global pattern was confirmed by  
313 DEG analyses carried out for each holobiont. H1 and H2 exhibited only a low number of  
314 differentially expressed genes (6 and 91, respectively; enriched in 0 and 6 GO categories,  
315 respectively), while in H3 2,355 genes (enriched in 100 GO terms) were differentially expressed  
316 ([Figure 5A](#)). A summary of the most important processes that were enriched among DEGs in  
317 each of the holobionts is given in [Table 2](#), and details are provided in [Supplementary Table S6](#).  
318 While H3 showed a more general response with several processes of primary metabolism being  
319 repressed in 15% NSW, H1 and H2 exhibited more specific responses. Interestingly processes  
320 classically associated with stress response, such as the production of heat shock proteins, were  
321 repressed in H3 low salinity.

322 A key objective of this work was to determine how the freshwater-intolerant holobiont  
323 (H3) differed from the freshwater-tolerant holobionts (H1+H2), both in terms of basal gene  
324 expression in 100% NSW and regarding its response to low salinity. This was accomplished by  
325 DEG analysis of the data grouping H1+H2 and using a two-factor model (holobiont\*salinity).  
326 In this model, genes significant for the factor holobiont correspond to basal differences in gene

327 expression between freshwater-tolerant and freshwater-intolerant algae. Overall, 10,059 genes  
328 fell in this category; 7,966 were down-regulated, and 2,093 were upregulated in H3.  
329 Overrepresented GO terms associated with these genes cover a wide range of primary metabolic  
330 and cellular processes and are listed in [Table 2](#) and, in more detail, in [Supplementary Table S6](#).

331 In the same model, genes significant for the interaction term correspond to holobiont-  
332 specific gene regulation differences in response to low salinity. Here 1,266 genes were  
333 upregulated explicitly in H3 low salinity, and 726 specifically down-regulated. The  
334 physiological processes and GO terms overrepresented among these genes are summarized in  
335 [Table 2](#) and listed in [Supplementary Table S6](#). They comprise several typical responses to  
336 changing salinity, such as transmembrane transport (overrepresented in both up- and down-  
337 regulated genes), or lipid metabolism with activation of lipid breakdown in H3 in low salinity.

### 338 [Microbial gene expression](#)

339 For the analysis of microbial gene expression patterns, despite the availability of a  
340 metagenome, a classical gene by gene and organism by organism analysis was not feasible due  
341 to the low final read coverage and the high number of microbes/microbial genes present (only  
342 0.7% of all reads mapped to the 73 microbial bins). Therefore, we used two alternative  
343 approaches to exploit the available data. First, we summarized gene expression data for all  
344 genes within a given metagenomic bin to determine each bin's overall transcriptomic activity.  
345 Secondly, we merged gene expression data for all genes predicted to catalyze the same  
346 metabolic reaction across all bins. These latter data were used to examine differences in the  
347 overall metabolic activity of the entire microbiome in the tested conditions.

348 Normalized transcriptional activity per bin was visualized in a heat map ([Figure 6](#)). It  
349 shows that each of the three holobionts is characterized by the transcriptomic activity of  
350 different metagenomic bins, and smaller differences within each of these clusters separate the

351 100% NSW from the 15% NSW conditions. A similar pattern was also observed in the PCA  
352 plot based on the microbiome's overall metabolic activity: again, holobiont was the main  
353 separating factor, but within each holobiont, separation according to the salinity treatment is  
354 visible (Figure 4B).

355 To determine the metabolic specificities of the bacteria associated with the tested  
356 holobionts and conditions, expression data for each of the 3,957 metabolic reactions  
357 (Supplementary Table S5) were subjected to differential expression analysis. Hundred nine  
358 significant reactions were identified in holobiont H1, 226 in holobiont H2, and 117 in holobiont  
359 H3 (Figure 5B, Supplementary Table S7). In the microbiome of holobiont H1, gene expression  
360 differences between the salinity levels concerned most importantly glycine biosynthesis  
361 (induced in low salinity) and quinone biosynthesis as well as quorum sensing (repressed in low  
362 salinity). In holobiont H2, glycine, sorbitol, butanol, and polyamine metabolism were induced,  
363 and several genes related to nucleotide degradation and quinone metabolism were repressed in  
364 low salinity (among other reactions). In holobiont H3, unlike in holobiont H1 and H2, osmolyte  
365 production (glycine-betaine, ectoine) was repressed in low salinity, along with some  
366 carbohydrate degradation pathways and ATP as well as NAD metabolism. No pathways in this  
367 holobiont contained >50% of induced reactions in low salinity (see Supplementary Table S7)  
368 for a complete list of reactions). Only one reaction, catalyzed by a diaminobutyrate  
369 aminotransferase (R101-RXN), was repressed in low salinity by all holobionts and no reactions  
370 were significantly upregulated in all holobionts. Comparing global expression patterns of  
371 holobiont H3 with holobionts H1 and H2 in full salinity highlighted only 4 pathways: hydrogen  
372 oxidation, phosphonoacetate degradation, pyruvate fermentation, and hydrogen to fumarate  
373 electron transfer, all of which were upregulated in holobiont H3.

374 Examining reactions significant for the interaction term, i.e. microbial reactions for  
375 which the response to low salinity differed between low-salinity tolerant and intolerant

376 holobionts, only one pathway, autoinducer AI-1 biosynthesis, emerged. This pathway was  
377 explicitly upregulated in the fresh water-intolerant holobiont H3 in low salinity conditions,  
378 notably by bacteria of the genera *Hoeflea* (bin 29), *Roseovarius* (bins 55 and 69), and  
379 *Sulfitobacter* (bin 5). The other 21 significant reactions did not constitute pathways with >50%  
380 of genes regulated, but, given their potential importance, they were manually grouped into 8  
381 metabolic categories: ectoine synthesis, phospholipids, phosphate metabolism, selenate  
382 reduction, seleno-amino acid biosynthesis, carbon metabolism, vitamins, and DNA repair, all  
383 of which were repressed or absent in holobiont H3 in low salinity (Table 3). Notably, the  
384 demethylphyloquinone reduction reaction (RXN-17007) constituting the last step of the  
385 biosynthetic pathway of vitamin K was expressed by three bins, although at very low levels.  
386 Bin 32 (unclassified bacterium) expressed this reaction in holobionts 2 and 3 in seawater, bin  
387 19 (*Hoeflea*) expressed the reaction in holobiont 1 (both seawater and 15% NSW conditions)  
388 and holobiont 3 in seawater, and bin 67 (*Halomonas*) expressed the reaction in holobionts 2 in  
389 15% NSW. However, none of the bacteria in our metagenome expressed RXN-17007 in H3 in  
390 15% NSW.

### 391 Expression of viral sequences

392 The analysis of viral sequences in the metatranscriptome revealed marked differences  
393 in viral abundances depending both on the holobiont and the culture condition (Figure 4C). The  
394 global patterns followed that of the algal transcriptome data, with holobiont 3 grouping apart  
395 from holobionts 1 and 2, and with a clear difference in viral composition in holobiont 3,  
396 depending on salinity. Holobiont 3 in 100% salinity conditions exhibited the highest relative  
397 abundance of viral sequences (Figure 7). The majority of annotated viral reads belonged to the  
398 *Phycodnaviridae*, dsDNA viruses known to infect algae (Wilson *et al.*, 2009). They were  
399 present in all samples and most abundant in H3-100%. The next most abundant groups of  
400 viruses were DNA phages belonging to the *Microviridae* and *Caudovirales*. These groups

401 specialize in bacterial hosts (King *et al.*, 2012; Doore and Fane, 2016). *Retroviridae* occurred  
402 specifically in holobiont 2 in 100% salinity, and of *Alloherpesviridae* also in holobiont 2 in  
403 100% salinity as well as in one replicate of H3-15%.

#### 404 **Metabolite profiling of algal holobionts**

405 The metabolite dataset contained 609 features that were (after normalization) used for  
406 statistical testing. In total, 72 features were significantly different in at least one of the tested  
407 conditions (Supplementary Table S8). Differences between H1 and H2 were negligible, with  
408 only one feature differing between the low and high salinity conditions of these two holobionts  
409 (a peak putatively corresponding to histidinol in H1, and a peak putatively corresponding to  
410 hydroquinone in H2 both down-regulated in low salinity). This was confirmed by cluster  
411 analysis (Figure 8), which does not separate these conditions. H3, however, exhibited  
412 differences in metabolic profiles depending on the salinity with 4 upregulated (quinic acid, 5-  
413 propionate-hydantoin, and two unknown features) and 35 down-regulated features in low  
414 salinity, including a feature annotated as phytol (peak #145, a precursor of vitamin K) and  
415 several primary metabolites such as galactoglycerol, glycine, alanine, valine, oxoproline. For  
416 all of these, the regulation was specific to H3. Furthermore, even under control conditions  
417 (100% NSW), metabolite profiles of H3 differed from holobionts H1 and H2, exhibiting higher  
418 abundances of 18 features in H3 compared to H1+H2 (including glycerol 3-P, pentafuranose,  
419 putrescin), and a lower abundance of 29 features (including alanine, galactoglycerol, and valine;  
420 see Figure 8).

#### 421 **Discussion**

422 This study aimed to identify bacteria and bacterial functions that could support *E.*  
423 *subulatus* holobionts to acclimate to low salinity. Three algal-bacterial holobionts that differed  
424 in their capacity to grow in low salinities were created. Our data show that variations in

425 microbiome impacted algal gene expression profiles and metabolomic features. Low salinity-  
426 tolerant holobionts were those that were given fewer antibiotics and had fewer differentially  
427 expressed genes and metabolites in response to changes in salinity. Our findings show that low  
428 salinity treatments were not particularly stressful for the algal host in freshwater-tolerant  
429 holobionts, despite microbiome changes resulting from the treatment. In the fresh water-  
430 intolerant holobiont, on the other hand, basal expression levels, metabolite profiles, and even  
431 viral expression levels were altered, and we observed a robust host response to salinity changes,  
432 similar to that described by Dittami *et al.* (2012). Algal holobionts were allowed to recover in  
433 antibiotic-free natural seawater medium (NSW) for weeks after the initial antibiotic treatment  
434 in our experiments. We, therefore, assume that the differences in the algal response to low  
435 salinity are related mainly to differences in the microbiome. This assumption is in line with the  
436 results of inoculation experiments demonstrating that low salinity tolerance in antibiotic-treated  
437 cultures has previously been restored by restoring the microbiome (Dittami *et al.*, 2016). Our  
438 data also suggest that viral transcription changes may further complexify this already complex  
439 system, as the relative abundance of viral reads varied depending on the holobiont and culture  
440 condition. These variations affected both viruses likely to infect the host and the associated  
441 bacteria. Such tri-partite trans-kingdom interactions have been described in mammalian guts  
442 (Pfeiffer and Virgin, 2016), but have, to our knowledge, not been considered in macroalgal  
443 research so far.

#### 444 [Three scenarios of microbial impacts on host stress tolerance](#)

445 Here we discuss the generated metatranscriptomic, metagenomic, and metabolomic data  
446 in the light of three different scenarios of how bacteria may impact algal low salinity tolerance:

- 447 1) Members of the microbiome of low salinity-tolerant holobionts may directly  
448 produce compounds such as osmolytes or chaperones that enhance algal low  
449 salinity tolerance or stimulate the alga to do so.
- 450 2) The microbiome provides essential services to the alga regardless of the salinity,  
451 but under stress, the microbiome of the low salinity-sensitive holobiont may no  
452 longer provide these services, ultimately leading to a reduction/stop in growth.
- 453 3) Under stressful conditions, the equilibrium in the microbiota may be disrupted,  
454 and certain microbes may proliferate and become harmful to the host. This  
455 phenomenon, termed dysbiosis, can be triggered both by algal and bacterial  
456 signals and possibly mitigated by the presence of other microbes or viruses.

#### 457 No clear signs of microbial contributions to algal stress response

458 In the first scenario, the alga-associated microbiome is assumed to produce compounds  
459 that enhance algal stress tolerance actively. In terrestrial environments, studies have  
460 highlighted, for instance, the microbial production of plant hormones, which activate plant  
461 defenses, making them more resistant against pathogens, or the uptake of nutrients (e.g. via the  
462 production of siderophores, that enhance the host ability to survive in low-nutrient  
463 environments) (Numan *et al.*, 2018). When examining the low salinity response of the different  
464 holobionts, we found little difference in algal gene expression or the examined metabolite  
465 profiles in the low-salinity tolerant holobionts. Furthermore, none of the observed changes  
466 corresponded to the induction of classical stress response genes.

467 On the bacterial side, the only low-salinity induced microbial pathway common to both  
468 low salinity-tolerant holobionts was glycine synthesis, but we did not detect significant changes  
469 in glycine concentrations in the tissues of these holobionts. Glycine has been shown to enhance  
470 growth in some microalgae, including diatoms (Berland *et al.*, 1979). However, no data is

471 available on the impact of external glycine on brown algal growth rates. Furthermore,  
472 *Ectocarpus* can synthesize glycine and does so primarily during the daytime (Gravot *et al.*,  
473 2010). This does not exclude a role of glycine or other bacterial compounds in the brown algal  
474 stress response, but our transcriptomic data provide little support for this hypothesis.

475 An alternative way microbes may increase host tolerance to stressors is by priming the host and  
476 activating its “defenses” even before exposure to stress – a process previously reported in kelps  
477 (Thomas *et al.*, 2011). Indeed, a comparison of transcriptomic and metabolic profiles of  
478 holobionts H1 and H2 vs H3 revealed fundamental differences, indicating that the host  
479 compartments of the different holobionts were not in the same physiological state at the start of  
480 the experiments. However, most of the processes upregulated in H1+H2 were related to the  
481 cytoskeleton and not GO categories such as “response to stimulus” or more specific sub-  
482 categories. Thus, while our results cannot exclude potential defense priming effects, the algal  
483 host's transcriptomic regulation does not support this hypothesis.

#### 484 [Loss of microbial services in non-tolerant holobionts: vitamin K](#)

485 A second scenario is that changes in the microbiome triggered by the salinity change  
486 result in a lack of microbial services essential for the alga. Bacteria are known to provide, e.g.  
487 growth hormones to brown algae, including *Ectocarpus* (Pedersén, 1973; Tapia *et al.*, 2016),  
488 and more profound metabolic interdependencies have been predicted based on metabolic  
489 networks (Burgunter-Delamare *et al.*, 2020). However, an exhaustive list of these microbial  
490 contributions is still missing. A loss of such benefits could result from shifts in the microbiome  
491 composition, changes in the activity of different microbes, or specific changes in bacterial gene  
492 expression patterns. Our transcriptomic and metabolomic data show that holobiont H3 had  
493 fundamentally different basal profiles even in seawater before applying any low salinity stress.  
494 Notably, several primary metabolic processes were activated compared to holobionts 1 and 2,

495 such as the synthesis of amino acids and lipids, photosynthesis, carbohydrate metabolism, and  
496 transcription/translation. In contrast, several primary metabolites were less abundant (proline,  
497 alanine, glycine, serine, citric acid, and more, [Figure 7](#)). One possible interpretation of these  
498 observations is that the host in holobiont H3 was able to compensate for the absence of bacterial  
499 functions as long as it was growing in seawater but was no longer able to do so when transferred  
500 to low salinity, resulting in a repression of primary metabolism and a reduction of growth.

501 Our transcriptomic data highlighted bacterial metabolic processes that were less  
502 expressed and possibly absent specifically in H3 in low salinity ([Table 3](#)). Based on the  
503 *Ectocarpus* genome (Cock *et al.*, 2010), most of these processes are likely to be achievable by  
504 the algal host itself without requiring input from bacteria (carbon, phosphate, selenate, and  
505 phospholipid metabolism, DNA repair). While this does not preclude that these compounds or  
506 activities may be helpful for the host, their provision from external sources is likely not  
507 essential.

508 In the same vein, the synthesis of ectoine was repressed in the bacterial  
509 metatranscriptome H3 in low salinity. Ectoine is known to serve as an osmolyte in bacteria  
510 (Czech *et al.*, 2018) and further studies are necessary to determine if ectoine might be also  
511 released to the environment to the benefit of the host. Microalgae without an associated  
512 microbiome contain ectoine in small amounts, pointing towards a dual origin of this metabolite  
513 in the algae from their own biosynthesis as well as from uptake (Fenizia *et al.*, 2020). However,  
514 ectoine might be only used as an osmolyte for bacteria in 100% NSW, and may no longer be  
515 required at low salinity.

516 Vitamin K is involved in the functioning of the photosystem in land plants. Mutants of  
517 *Arabidopsis* and *Cyanobacteria* missing the last reaction of its biosynthetic pathway are viable  
518 but exhibit increased photosensitivity (Fatihi *et al.*, 2015). In *E. subulatus*, just as in *E.*  
519 *siliculosus*, *Saccharina latissima*, and *Cladosiphon okamuranus* (Nègre *et al.*, 2019; Dittami,

520 Corre, *et al.*, 2020), the last step of the vitamin K biosynthetic pathway is absent from the algal  
521 metabolic network. It suggests that these algae cannot produce vitamin K independently,  
522 although this compound has previously been detected in kelps (Yu *et al.*, 2018). If, as suggested  
523 by our transcriptomic and metabolic data, bacterial vitamin K production is repressed  
524 specifically in H3 in low salinity, it is plausible that the resulting lack of vitamin K may  
525 negatively impact algal growth. This constitutes a promising hypothesis to be tested, for  
526 instance via complementation experiments.

### 527 [Indications for dysbiosis](#)

528 The last of the three scenarios assumes that, in the fresh water intolerant holobiont, a  
529 change in salinity led to a change in the bacterial community or activity, notably the propagation  
530 of specific microbial strains that harm the host (dysbiosis). Dysbiosis is a well-studied  
531 phenomenon, especially in mammalian models. It is known to be the basis of several diseases  
532 (e.g. Hawrelak and Myers 2004) and assumed to be widespread also in marine environments  
533 (Egan and Gardiner, 2016).

534 In our data, we observed a significant induction of the AI-1 pathway, specifically in H3  
535 in low salinity (i.e. in the interaction term), supporting the hypothesis of the activation of  
536 quorum sensing compounds. Quorum sensing (QS) and AI-1, in particular, have been linked to  
537 dysbiosis in several model systems. For instance, in the coral *Pocillopora*, AI-1 type QS  
538 compounds have been related to coral bleaching (Zhou *et al.*, 2020). Similarly, in *Acropora*,  
539 inhibitors of AI-1 have been shown to prevent white band disease (Certner and Vollmer, 2018).  
540 In several microalgae, QS molecules have also been associated with the bacterial production of  
541 algicidal proteins or compounds such as proteases, amylases, quinones, or in many cases,  
542 unknown compounds (Paul and Pohnert, 2011; Demuez *et al.*, 2015). Furthermore, we  
543 observed a 4-fold increase in the relative bacterial ‘activity/abundance’ as measured by the ratio

544 of bacterial mRNA to algal mRNA reads, suggesting strong bacterial proliferation as typical  
545 for dysbiosis. This increase in transcription levels concerned primarily bacteria that were little  
546 active in the other conditions (unclassified *Bacteroidetes*, *Hyphomonas*, *Sphingorhabdus*,  
547 *Roseovarius*, unclassified *Rhizobiales*, *Erythrobacter*, *Brevundimonas*, Figure 6).

548 Both observations, the induction of QS genes and the increase in bacterial abundance,  
549 fit well with the scenario of dysbiosis. However, there are two significant limitations: First, QS  
550 compounds are not exclusively linked to dysbiosis and virulence – they may also be involved  
551 in processes such as the regulation of bacterial motility, biofilm formation, or the regulation of  
552 nutrient uptake (Zhou *et al.*, 2016), and in our data, we did not observe the induction of  
553 virulence-related genes. This could be explained by the nature of our analyses, which considers  
554 only genes with known functions that are represented in the MetaCyc database. Any (unknown)  
555 compounds or genes not represented in MetaCyc would not have been detected. The second  
556 limitation is linked to the first. Without further targeted experiments, it is not possible to assert  
557 if the induction of QS genes may be an indirect cause of the poor algal physiological state, or  
558 rather if the behavioral response of the bacteria to this compound causes poor algal health.  
559 Based on these observations, we believe that the role of QS during freshwater acclimation may  
560 be of interest for future studies, e.g. with QS inhibitors, and these studies should also  
561 specifically examine the production of potential virulence factors.

## 562 Conclusion

563 The case of the freshwater strain of *E. subulatus* and its reliance on its microbiome for  
564 growth and survival in freshwater is an excellent example of the importance of host-symbiont  
565 interactions. The system also demonstrates the difficulties that arise when attempting to  
566 understand the exact interactions in such complex systems, especially when they are not well-  
567 established models: fully controlled experiments to elucidate the specific functions are not (yet)

568 possible because we have not (yet) been able to cultivate or identify the right mix of bacteria  
569 required for freshwater tolerance. As a result, we chose to empirically modify holobiont  
570 composition and then study its behavior using a combination of metabolomics,  
571 metatranscriptomics, and metagenomics. Metabolic networks were used as a filter to combine  
572 and interpret the resulting complex datasets. This data only covers a portion of the biology of  
573 the organisms studied, but in our case suggested potential metabolic roles of associated bacteria  
574 in the supply of vitamin K, as well as a possible role of quorum sensing compounds in the  
575 holobionts that no longer exhibited growth - both hypotheses that can now be tested in targeted  
576 experiments, such as by supplementing vitamin K to the culture medium or by using inhibitors  
577 of quorum sensing. Lastly, our findings highlight the importance of considering viruses as a  
578 potential factor influencing holobiont acclimation to environmental change by interacting with  
579 both the host and the bacterial microbiome.

## 580 [Competing interests](#)

581 The authors declare that they have no conflict of interests.

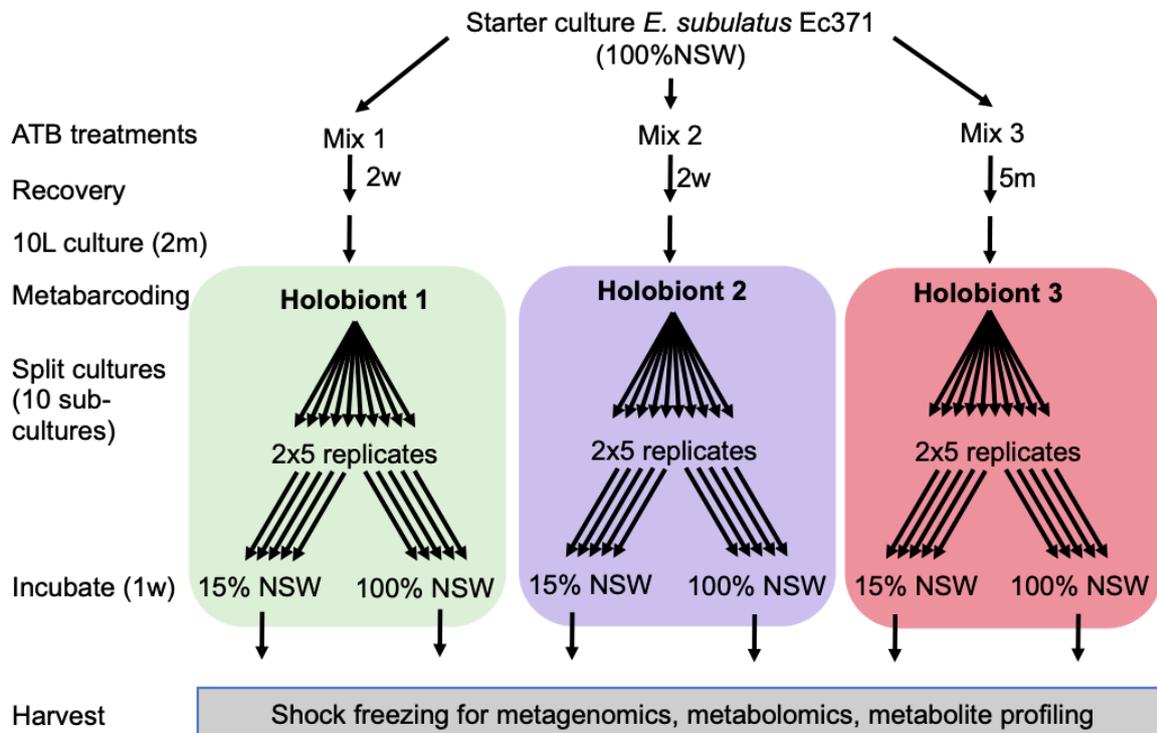
## 582 [Acknowledgments](#)

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594 **Figures**

595



596

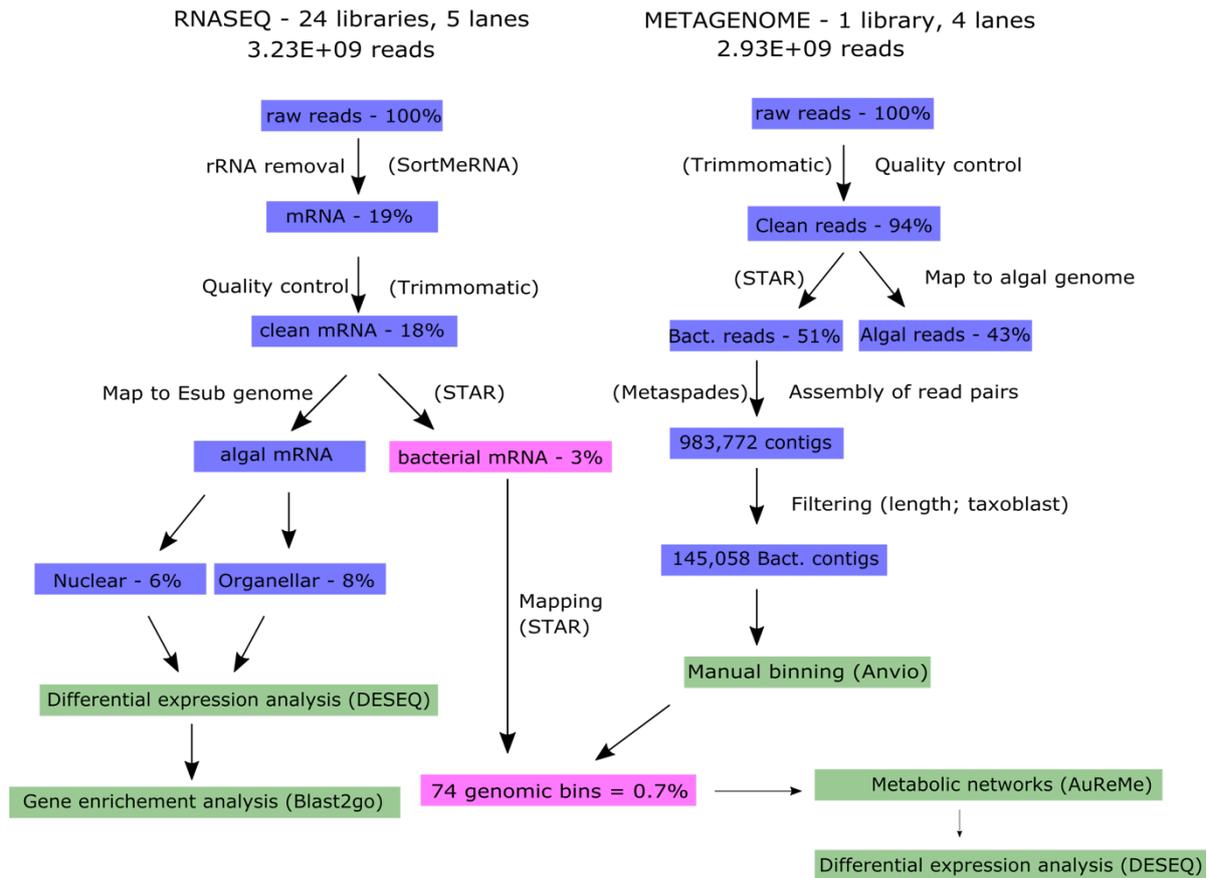
597 **Figure 1:** Overview of the experimental setup. All conditions were derived from the same  
598 starter culture, but antibiotic (ATB) treatments were carried out with three different ATB mixes  
599 (see methods) leaving hosts with different microbial communities, termed “holobionts 1-3”.  
600 The low-salinity response of each of these holobionts was then examined with 5 replicates.  
601 100% NSW=natural seawater, 15% NSW=15% NSW in distilled H<sub>2</sub>O (volume/volume),  
602 m=month(s), w=week(s).

603

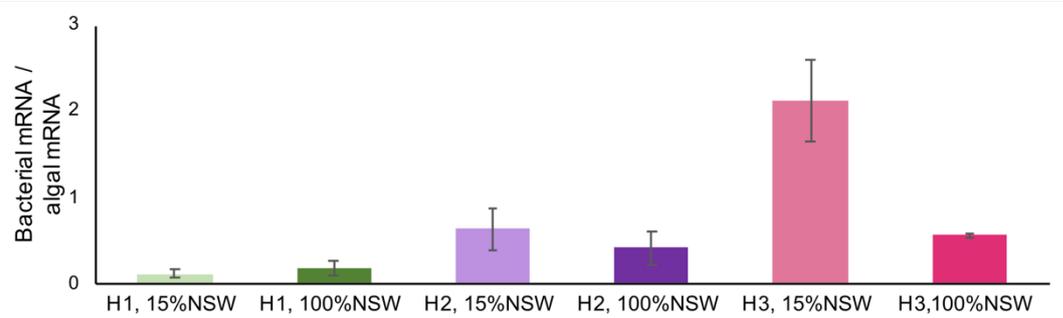
604

605

**A**



**B**



606

607 **Figure 2:** A) Overview of metatranscriptomic and metagenomic data obtained and the analysis

608 pipeline. The percentages correspond to the percentage of total raw reads at the start. B) ratio

609 of bacterial to algal mRNA in the different holobionts/conditions (mean of 4 replicates ± SD).

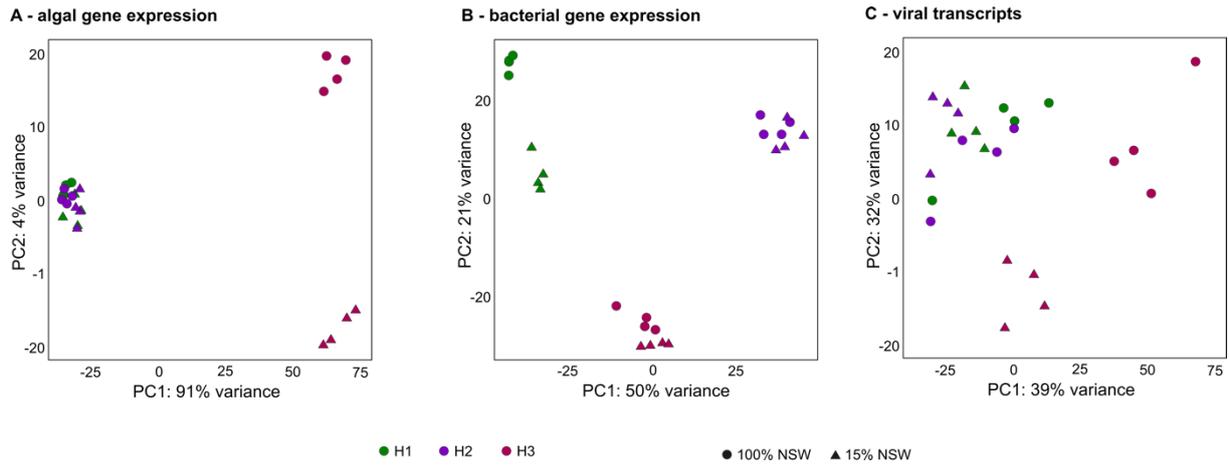
610 H3-15% is significantly different from all other conditions ( $p < 0.001$  Tukey HSD test.)

611

612

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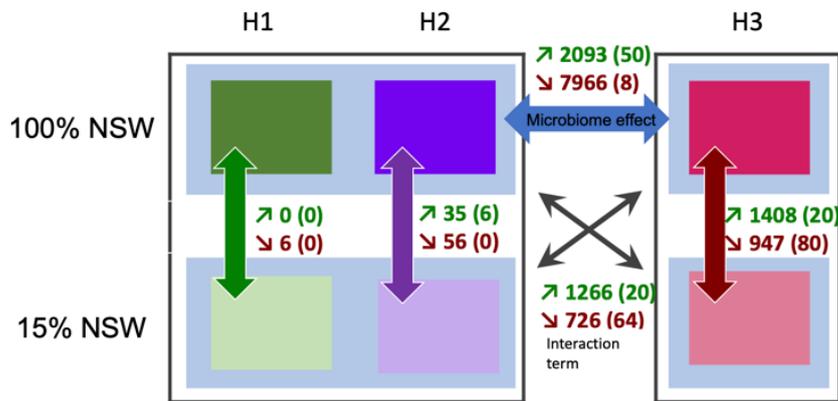
622

623 **Figure 4:** A) Principal component analysis (PCA) of algal gene expression. B) PCA of

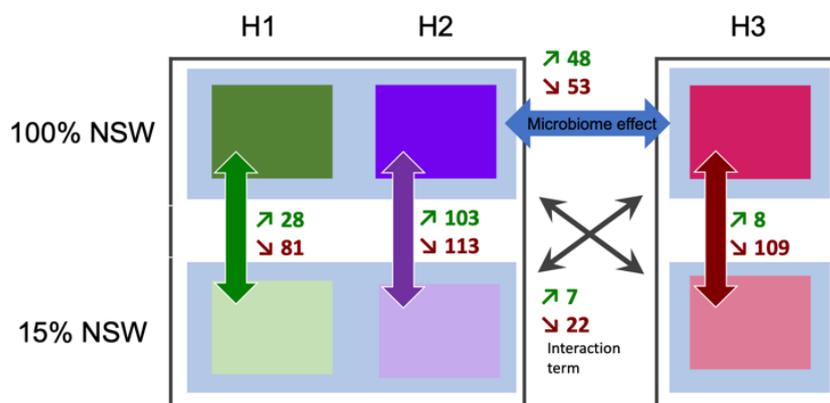
624 expression of bacterial reactions. C) PCA of viral transcripts

625

### A – algal gene expression

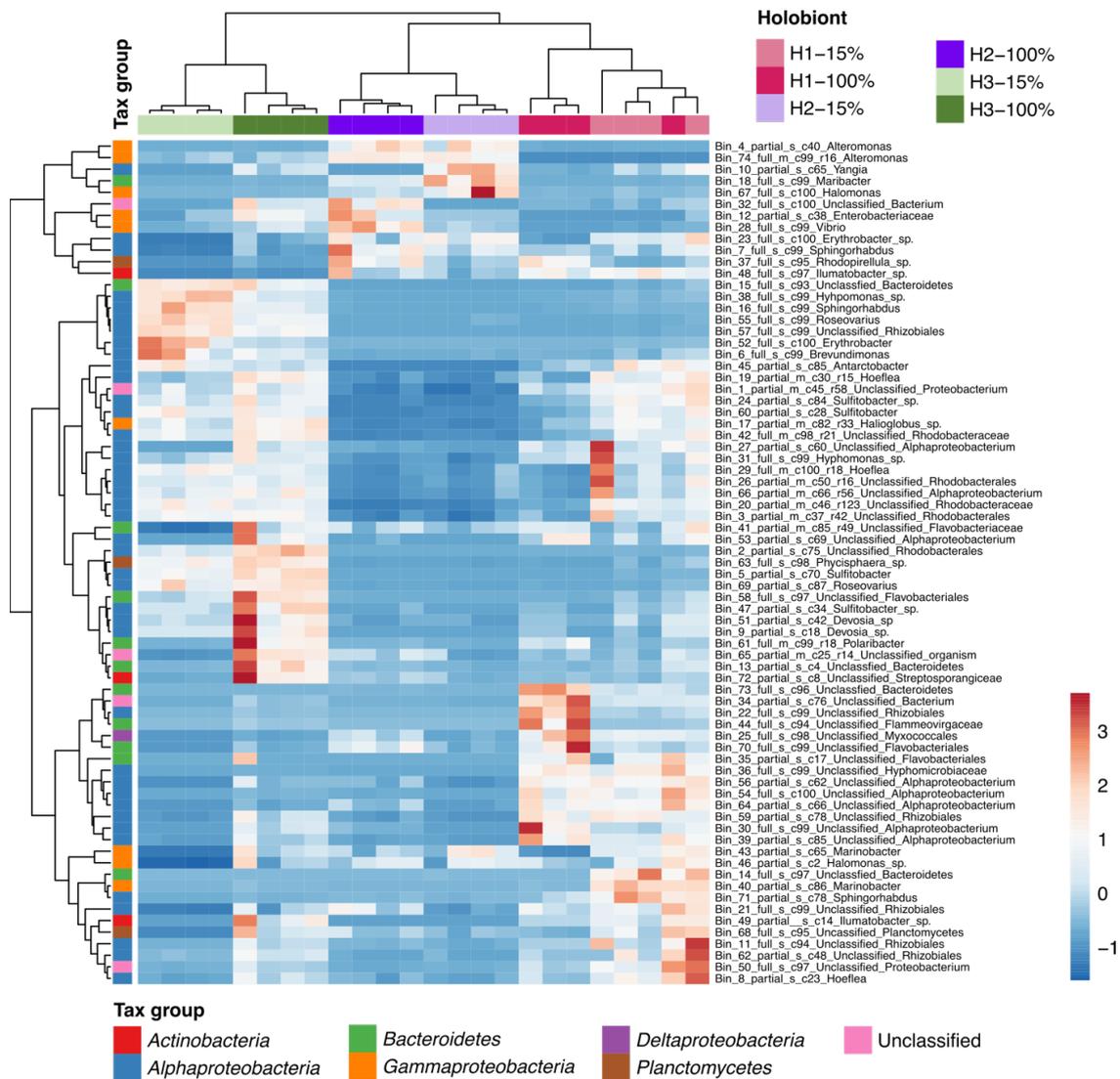


### B – bacterial gene expression (metabolic reactions)



626

627 **Figure 5:** A) Differentially expressed algal genes. The figure shows the number of differentially  
 628 expressed algal genes in each of the holobionts in 15% NSW compared to 100% NSW (H1,  
 629 H2, and H3); H1 + H2 jointly compared to H3 in 100% NSW (microbiome effect), and the  
 630 difference in the low salinity-response of H3 compared to that of H1+ H2 (interaction term,  
 631 crossed arrows). Numbers in parentheses correspond to the number of overrepresented GO  
 632 terms associated with the differentially regulated genes. B) Similar analysis as A, but on the  
 633 bacterial transcriptome; in this case, the analysis was based on differentially expressed  
 634 metabolic reactions rather than genes.



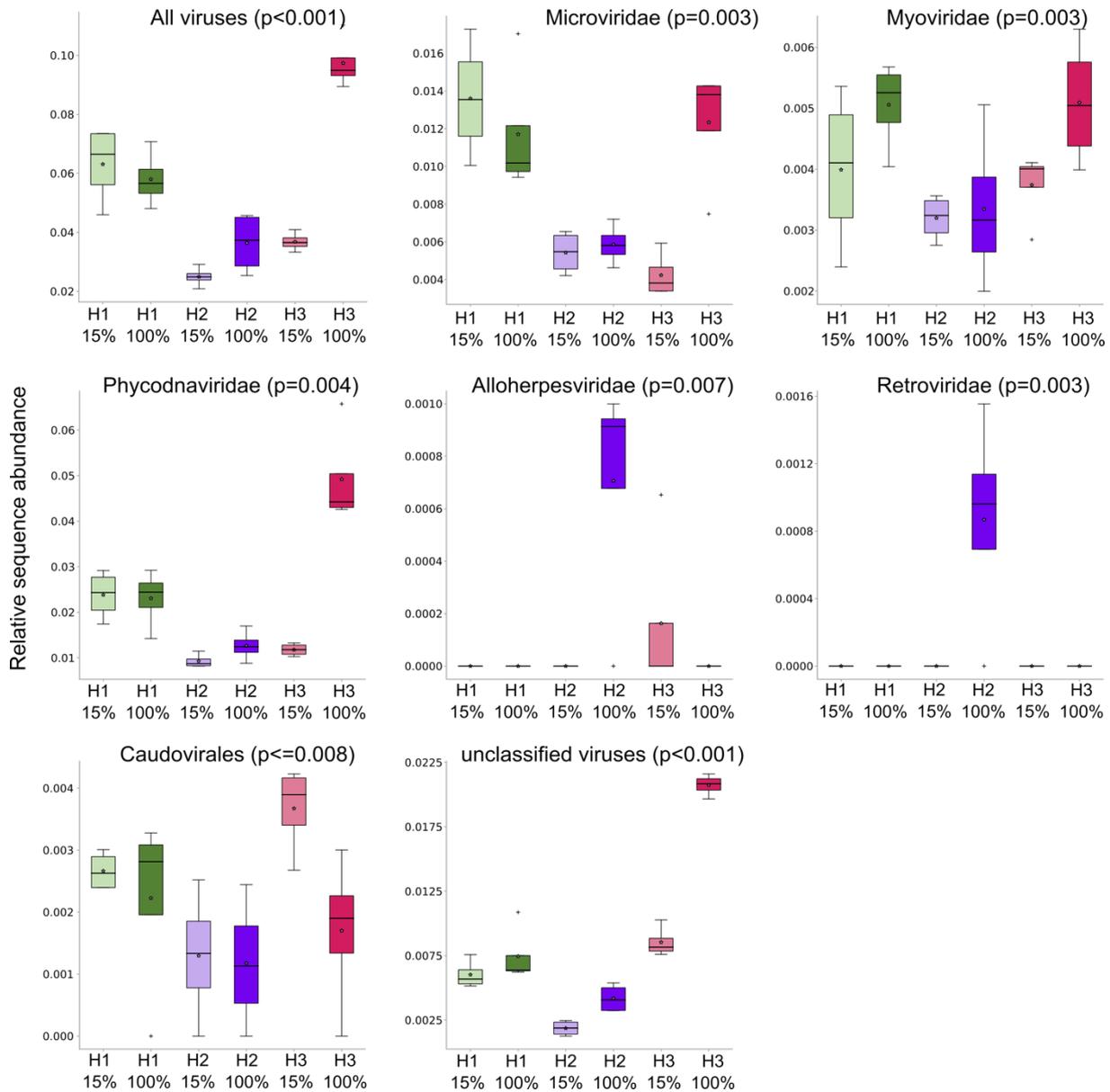
635

636 **Figure 6:** Heatmap based on hierarchical clustering of normalized transcriptional activity per  
 637 bacterial bin in each holobiont and condition (Pearson correlation coefficient; clustering  
 638 method: average linkage; unit variance scaling of row data). Red indicates high relative  
 639 transcriptomic activity in the given condition, blue low activity. “15%” corresponds to the  
 640 treatment with 15% natural seawater (NSW), “100%” to the treatment with 100% NSW. Bins  
 641 labeled “full” are predicted  $\geq 90\%$  complete, bins labeled “partial”  $<90\%$ . The predicted  
 642 completeness in % is given after the “c” in the bin name.

643

644

645

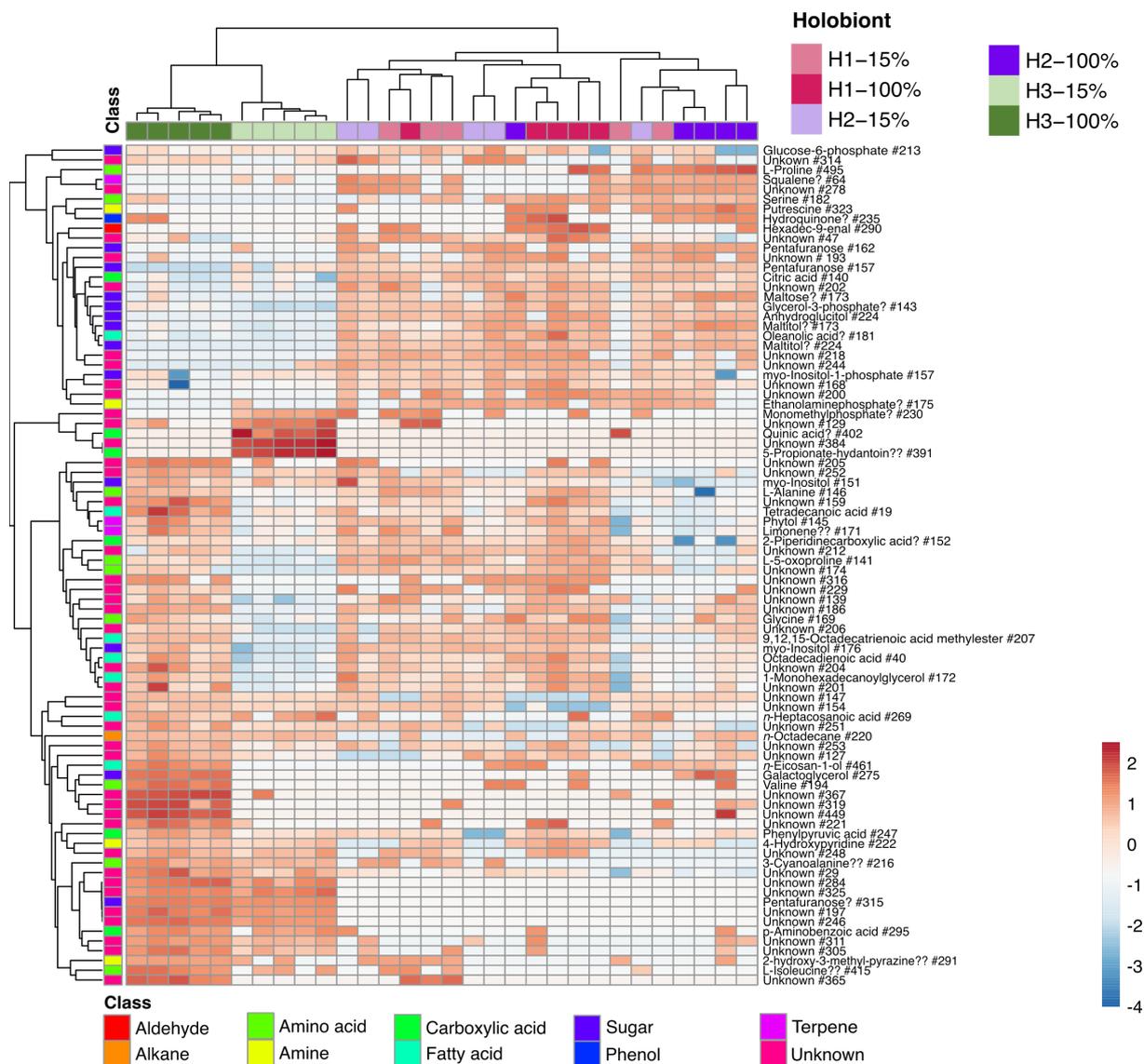


646

647 **Figure 7:** Box plot of relative abundances of viral sequences belonging to different families in  
648 all samples (H1-H3: holobiont 1-3). Values are given in % of the total number of RNAseq reads  
649 for each sample. P-values correspond to the results of an ANOVA across all six conditions (4  
650 replicates each). 15% corresponds to the treatment with 15% NSW, 100% to the treatment with  
651 100% NSW.

652

653



654

655 **Figure 8:** Heat map based on the abundance of each of the metabolites tested as significant in  
 656 at least one condition (Pearson correlation coefficient; clustering method: average linkage; unit  
 657 variance scaling of row data). 15% corresponds to the treatment with 15% NSW, 100% to the  
 658 treatment with 100% NSW. “?” indicates features with reverse match score (R)<800, “??”  
 659 features with R<700. Features were labeled “unknown” for R<600.

660

661 **Tables**

662 **Table 1:** Different algal holobionts used for the metatranscriptomics/metabolomics experiment;  
663 Holobiont H1: treated with rifampicin, penicillin and neomycin (each 100 µg/ml); Holobiont  
664 H2: idem, plus streptomycin (25 µg/ml) and chloramphenicol (5 µg/ml); Holobiont H3: treated  
665 with penicillin (12000UI), Chloramphenicol (0.75 µg/ml), Polymyxin B (0.75 µg/ml),  
666 Neomycin (0.9 µg/ml). The untreated holobiont (H0) was only used for 16S rRNA gene  
667 metabarcoding, not for metatranscriptomics/metabolomics. Algae were considered “alive” as  
668 long as their pigmentation was visible, even in the absence of visible growth.

669

	<b>Full microbiome</b>		<b>Modified microbiomes</b>	
Label	H0	H1	H2	H3
No. of antibiotics	0	3	5	4
Type of treatment	n/a	solution	solution	discs
Exposure time	n/a	3 days	3 days	5 weeks
Bacterial growth plates	Yes	No	No	No
Survival in 100% NSW	Yes, growth	Yes, growth	Yes, growth	Yes, growth
Survival in 15% NSW	Yes, growth	Yes, growth	Yes, growth	Yes, no visible growth
Survival in 5% NSW	Yes, growth	Yes, growth	Yes, growth	No

670

671 **Table 2:** Summary of the differentially regulated processes during the response to low salinity  
 672 in the algal host of each holobiont based on the summary of enriched GO terms from  
 673 [Supplementary Table S6](#). Upregulated genes are significantly induced in 15% NSW compared  
 674 to 100% NSW and/or in holobiont H3 compared to holobiont 1+2 (interaction term).  
 675 Downregulated refers to genes significantly repressed in the same conditions.  
 676

	<b>Upregulated in 100% or H3</b>	<b>Downregulated in 100% or H3</b>
<b>Holobiont H1</b> 100% NSW vs. 15% NSW		heat shock protein SAM-dependent methyltransferase dynein heavy chain protein
<b>Holobiont H2</b> 100% NSW vs. 15% NSW	LHCR/LHCF Cytochrome / PSII (cp) Ribosomal proteins (cp) Transcription / Translation	C5-epimerase heat shock protein 70
<b>Holobiont H3</b> 100% NSW vs. 15% NSW	Ammonium transmembrane transport Mannose synthesis	Nitrate assimilation Photosynthesis Amino acid metabolism Vitamin biosynthesis Lipid metabolism
<b>Holobiont H1+H2</b> vs. <b>Holobiont H3</b>	Photosynthesis Transmembrane transport Transcription / translation Amino acid metabolism Lipid metabolism Carbohydrate metabolism Nitrogen metabolism	Cytoskeleton
<b>Interaction term</b>	Transmembrane transport	Amino acid metabolism Lipid metabolism Photosynthesis Vitamins Carbohydrate metabolism

677

678 **Table 3:** Metabolic reactions with different low salinity responses in H3 compared to H1 and  
 679 H2 (interaction term). The first column contains the MetaCyc reaction ID and, in parentheses,  
 680 the corresponding enzyme. If the same enzymes carried out several (similar) reactions, these  
 681 were grouped and only one representative is shown. If different than 1, the total number of  
 682 reactions catalyzed by this enzyme is given in parentheses following an x after the enzyme  
 683 name. “Induced” means that this reaction was more strongly upregulated in H3 in response to  
 684 15% NSW than in H1 and H2. Repressed or absent means the reaction was downregulated or  
 685 absent in H3 in 15% NSW.

	<b>Regulation</b>	<b>Important taxa</b>
<b>Quorum sensing</b>		
2.3.1.184-RXN (acyl-homoserine-lactone synthase) (x6)	Induced	<i>Sulfitobacter</i> , <i>Roseovarius</i> , <i>Hoeflea</i>
<b>Ectoine synthesis</b>		
R101-RXN (diaminobutyrate aminotransferase)	Repressed	<i>Antarctobacter</i> , <i>Roseovarius</i> , <i>Halomonas</i>
R102-RXN (diaminobutanoate acetyltransferase)	Repressed	<i>Antarctobacter</i> , <i>Roseovarius</i> , <i>Halomonas</i>
R103-RXN (ectoine synthase)	Repressed	<i>Antarctobacter</i> , <i>Roseovarius</i> , <i>Halomonas</i>
<b>Phospholipids</b>		
GLYCPDIESTER-RXN (glycerophosphoryl diester phosphodiesterase) (x3)	Repressed	<i>Antarctobacter</i> , <i>Alteromonas</i> , <i>Sphingorhabdus</i> , <i>Sulfitobacter</i> , <i>Erythrobacter</i> , uncl. <i>Rhizobiales</i>
LIPIDXSYNTHESIS-RXN (UDP-2,3-diacylglucosamine diphosphatase)	Repressed	<i>Hoeflea</i> , <i>Phycisphaera</i> sp., uncl. <i>Bacteroidetes</i>
<b>Phosphate turnover/metabolism</b>		
3.11.1.2-RXN (phosphonoacetate hydrolase)	Repressed	<i>Hoeflea</i>
ABC-27-RXN (phosphate ABC transporter)	Repressed	<i>Sphingorhabdus</i> , <i>Rhodobacteraceae</i> , <i>Hoeflea</i> , <i>Antarctobacter</i> , <i>Erythrobacter</i> , <i>Rhizobiales</i> , <i>Phycisphaera</i> , <i>Alteromonas</i>
RXN0-1401 (ribose 1,5-bisphosphate phosphokinase)	Repressed	<i>Halomonas</i> , <i>Hoeflea</i> , uncl. <i>Rhizobiales</i> , <i>Antarctobacter</i>
<b>Selenate reduction/seleno-amino acid biosynthesis</b>		
RXN-12720 (ATP-sulfurylase) (x2)	Repressed	<i>Illumatobacter</i> , uncl. bacterium, uncl. <i>Bacteroidetes</i> , <i>Alteromonas</i>
RXN-12730 (5-methyltetrahydropteroyltri-glutamate--homocysteine S-methyltransferase)	Repressed	<i>Halomonas</i>
<b>Carbon metabolism</b>		
RXN-961 (ribulose bisphosphate carboxylase/oxygenase)	Repressed	Uncl. bacterium, <i>Algoriphagus</i> , <i>Lutibacter</i>

2.8.3.22-RXN (Succinyl-CoA--L-malate CoA-transferase) (x4)	Repressed	<i>Sphingorhabdus</i> , <i>Hoeflea</i> , uncl. <i>Rhodobacterales</i> , <i>Hyphomonas</i>
RXN-14365 (D-psicose 3-epimerase.)	Repressed	<i>Hoeflea</i> , <i>Antarcticobacter</i>
<b>Vitamins</b>		
RXN-17007 (Demethylphyloquinone reductase)	Absent	<i>Hoeflea</i> , <i>Halomonas</i> , uncl. bacterium
<b>DNA repair</b>		
3.1.21.2-RXN (Endonuclease)	Repressed	<i>Hoeflea</i>

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