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## **An invasive herbivorous fish (*Siganus rivulatus*) influences both benthic and planktonic microbes through defecation and nutrient excretion**

Arthur Escalas, Amandine Avouac, Jonathan Belmaker, Thierry Bouvier, Valentin Clédassou, Franck Ferraton, Fabien Rieuvilleneuve, Gil Rilov, Anna Rovirosa Mulet, Daphna Shapiro Goldberg, et al.

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1 **An invasive herbivorous fish (*Siganus rivulatus*) influences both benthic and planktonic microbes**  
2 **through defecation and nutrient excretion**

3  
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14  
15  
16 **ABSTRACT**

17 Direct and indirect impacts by invasive animals on plants and other animals through predation and competition  
18 have been evidenced in many ecosystems. For instance, the rabbitfish *Siganus rivulatus*, originating from the  
19 Red Sea, is now the most abundant species in coastal habitats of South-Eastern Mediterranean Sea where it  
20 overgrazes algae. However, little is known about its impacts on microbes through release of metabolic wastes  
21 and feces. We used a mesocosm experiment to test the effect of *S. rivulatus* on planktonic and benthic microbial  
22 communities. Excretion of dissolved nutrients by fish resulted in higher concentrations of dissolved inorganic  
23 nitrogen (NH<sub>4</sub>, NO<sub>2</sub>/NO<sub>3</sub>). This increase in availability of N was associated with higher N content in  
24 macroalgae, higher biomass of phytoplankton, higher abundance of bacterioplankton and shift in the structure  
25 of planktonic bacterial communities. The feces released mostly under the shelters where the fish rest at night,  
26 led to significant increases in diversity of sediment bacterial communities and shifts in their structure. The  
27 impact of *S. rivulatus* on planktonic microbes was related to the indirect bottom-up effect induced by excreted  
28 dissolved nutrients while its effect on benthic microbes was due to the direct release of both organic matter  
29 and microbes present in feces. Overall, this first evidence of the impacts of invasive species on planktonic and  
30 benthic microbes highlights that ongoing changes in fish biodiversity could have ecosystem-wide  
31 consequences.

32  
33 **Keywords:** biological invasion, Lessepsian species, nutrient recycling, nutrient excretion, microbiome, coastal  
34 marine ecosystem, *Siganus rivulatus*

## 37 I. INTRODUCTION

38

39 Nutrient cycling is a key biogeochemical process for functioning of all ecosystems (Elser et al., 2007). In  
40 marine coastal ecosystems, primary productivity is driven by the availability of dissolved and/or particulate  
41 forms of nutrients such as nitrogen (N) or phosphorus (P). Many organisms compete for these nutrients, from  
42 the planktonic and benthic prokaryotes, to uni- and multi-cellular eukaryotes such as phytoplankton,  
43 macroalgae and seagrasses (Philippart et al., 2007; Sundareshwar et al., 2003; Zaldivar et al., 2009). Although  
44 most of the nutrient cycling is mediated by the microbial loop (Pomeroy et al., 2007), macro-organisms, and  
45 especially fish, are increasingly recognized as key contributors to nutrient recycling in coastal ecosystems  
46 (Allgeier et al., 2017, 2013). Herbivorous fishes constitute the dominant trophic guild in sub-tropical and  
47 tropical reef ecosystems, where they exert a significant top-down control on the abundance of macrophytes  
48 through grazing (*e.g.* Bellwood et al., 2004; Dell et al., 2020).

49 In addition, herbivorous fishes influence nutrient dynamics in aquatic ecosystems through consumer-driven  
50 nutrient dynamics (Capps & Flecker 2013; Burkepile et al 2013). Indeed, all fish excrete ammonia (NH<sub>4</sub><sup>+</sup>) and  
51 phosphate (PO<sub>4</sub>) as dissolved metabolic wastes (Vanni 2002; Ip and Chew, 2010). Nutrient fluxes produced by  
52 fish communities reach levels comparable with other biogeochemical processes such as nitrogen fixation  
53 (Meyer and Schultz, 1985; Allgeier et al., 2017). Thus, in coral reefs, the nutrient excretion by fish could  
54 increase the nutrient content of benthic organisms such as corals and macroalgae (Burkepile et al., 2013, Shantz  
55 et al 2015). In addition, nutrient excretion by carnivorous fish can increase the abundance of both planktonic  
56 microalgae and bacteria and modify their diversity by favoring some taxa (Schaus & Vanni 2000, Villéger et  
57 al., 2019). However, there is currently no assessment of the effect of nutrient excretion by herbivorous fish on  
58 multiple compartments of marine ecosystems such as macroalgae, bacterioplankton and phytoplankton.

59 Fishes also recycle nutrients through the egestion of feces that are rich in particulate forms of organic nitrogen  
60 and phosphorous. For instance, the feces of carnivorous fishes often have a higher nutritional value than the  
61 regular food of herbivorous fishes, and can support the existence of fecal foodwebs (Robertson, 1982).  
62 Herbivorous fishes counterbalance their nutrient poor diet by eating continuously throughout the day and thus  
63 tend to defecate several times per day (Vermeij et al., 2013), which results in the recycling of large amounts of  
64 nutrients through feces deposition. These amounts could be especially high in refugia and sleeping areas (Bray  
65 et al., 1981), where they could stimulate benthic microbes because of their nutrient content. However, the effect  
66 of fish feces on benthic microbes has only been studied in the context of marine aquaculture (Quero et al.,  
67 2020), where fish densities exceed those observed in the natural environment. Fish can also influence  
68 environmental microbial communities as feces contain high abundance of active bacteria originating from the  
69 gut microbiome (Smriga et al., 2010; Troussellier et al., 2017). However, the influence of release of fecal  
70 microbes on the diversity and structure of environmental microbial communities remains to be tested.

71 Because of accelerating ocean warming and biotic exchange, marine herbivorous fishes have been colonizing  
72 new ecosystems in several regions for the last decades, contributing to marked decrease in macroalgae  
73 coverage with cascading effect on other organisms (Vergés et al., 2014, Vergés et al., 2019). Such  
74 tropicalization of temperate fish assemblages is particularly pronounced in the South-Eastern basin of the  
75 Mediterranean Sea where the opening of the Suez Canal serves as an entry point for tropical fishes from the  
76 Red Sea. Among those “Lessepsian” species, two herbivorous rabbitfishes (*Siganus rivulatus* and *S. luridus*)  
77 are the most successful colonizers with currently distribution spanning from the Levantine Sea to the north of  
78 the Aegean Sea and to the Sicily strait (Sala et al., 2011; Vergés et al., 2014). *Siganus spp.* have become highly  
79 dominant on shallow ecosystems of the Levantine Sea region (representing >90% of herbivorous fish biomass  
80 over rocky habitats; Frid et al., 2022), while the formerly only abundant native herbivorous fish (*Sarpa salpa*)  
81 is now very scarce (Rilov et al., 2018; Yeruham et al., 2020). *Siganus* have been overgrazing macroalgae to  
82 the point that ecosystems are now dominated by turf (Sala et al., 2011; Rilov et al., 2018; Peleg et al., 2019).  
83 However, while the effects of *Siganus spp.* on biomass and diversity of macrophytes and associated  
84 macrofauna have been assessed (Sala et al., 2011; Vergés et al., 2014), their impact on planktonic and benthic  
85 microbial communities still remain unknown.

86 In this study, we used an experimental approach in mesocosms to test three hypotheses: (i) the excretion of  
87 nutrients by *Siganus rivulatus* increases the nutrient content in macroalgae not grazed; (ii) the excretion of  
88 nutrients by *Siganus rivulatus* increases the abundance of planktonic bacteria and microalgae and modifies the  
89 diversity of their communities; (iii) the egestion of feces by *S. rivulatus* modifies the diversity of benthic  
90 microbial communities, especially under their resting spots.

91

92

## 93 **II. MATERIAL AND METHODS**

94

### 95 **II.1. Experimental design**

#### 96 *II.1.1- Mesocosm setup*

97 The experiment took place in the IOLR institute in Haifa (Israel), between April and June 2019 (Figure S1).  
98 Ten 800L cube-shaped and opaque plastic tanks were setup with 2 rows of 5 tanks (8 tanks for the experiment  
99 and 2 for fish acclimatization). The whole experimental area was shaded by a tarpaulin to reduce exposure to  
100 sunlight. Each tank was individually covered with a net (mesh size 2mm) to avoid direct sunlight into the  
101 mesocosms. On the 2<sup>nd</sup> of April 2019, the tanks were connected to the ILOR water circulation system and filled  
102 with seawater pumped less than 200m from the shore and water was renewed through overflow. The same day  
103 a 2cm-thick layer of sediment collected underwater from the shallow coastal habitat was added to each tank.  
104 On the night of 23<sup>rd</sup> of April, 34 *Siganus rivulatus* individuals were collected by hand during their sleep from  
105 the nearby reef by scuba divers and placed in two acclimation tanks. Two cinder blocks were placed on the

106 bottom of each acclimation tank to serve as shelter for the fish to reduce stress during acclimation. Fish were  
107 fed with macroalgae (mostly *Ulva* spp) collected in the nearby shoreline.

108 After two weeks of acclimation, the experiment started on the 7<sup>th</sup> of May 2019 (hereafter D0). The eight  
109 experimental tanks were set up to mimic as much as possible the natural habitat observed in the nearby shallow  
110 water. Three shelters were set up within each tank, so that *Siganus* could rest under them as they use to do with  
111 anfractuosity on reefs (Pickholz et al., 2018). Each shelter was made of two 20x20cm tiles with the bottom tile  
112 covered by a layer of sediment and the top tile was on two small rocks (Figure S1). Two shelters were left open  
113 to allow the fish to hide underneath while the third shelter was placed in a mesh bag so it was not accessible  
114 to fish (hereafter closed shelter), and corresponded to a control where the influence of the fish on the bottom  
115 sediment could be only through nutrient excreted in the water not through egestion of feces.

116 Four species of macroalgae that are abundant in the environment at this period of the year in the nearby shallow  
117 habitat were added to serve as food for the fish: *Ulva enteromorpha*, *Treptacantha rayssiae*, *Padina pavonica*  
118 and *Halopteris scoparia*. Each tank contained approximately 200g of *T. rayssiae*, 50g of *P. pavonica* and 50g  
119 of *H. scoparia*. As *U. enteromorpha* tend to float, we collected rocks covered with a tuft of this algae and  
120 placed them in approximately similar volume in each tank. Nine rocks covered with epilithic algal matrix  
121 (hereafter turf) were also added as *S. rivulatus* is also known to graze on turf (Lipkin and Lipkin, 1979). The  
122 densities of putative food sources present in each tank at the beginning of the experiment were similar to the  
123 one observed in the nearby habitat.

124 On the evening of the 7<sup>th</sup> of May, three fish individuals were added in four tanks (F1, F3, F5 and F7) for a total  
125 biomass of 100g (97-105g, Table S1) similar to the one observed in the shallow habitats were *Siganus* are the  
126 most abundant (Peleg et al., 2019). The four remaining tanks (C2, C4, C6 and C8) served as controls (no fish  
127 were added). Water renewal was stopped right after the introduction of the fish into the tanks. Ambient air was  
128 diffused through an air stone in each tank.

129 The experiment lasted for eight days, until the evening of 15<sup>th</sup> of May (D8) when the fish were removed from  
130 the tanks using handnet.

131 Fish behavior and the consumption of algae in the fish tanks was visually monitored daily to ensure that fish  
132 were acting normally and actively feeding as observed in the wild during this time of year.

133

134 *II.1.2- Samples collection*

135 Four types of samples were collected at the start (D0; before fish introduction) and the end of the experiment  
136 (D8; before fish removal): water, sediment, turf and macroalgae.

137 Water samples were collected in triplicates in each mesocosm at D0 and D8, and consisted of 1L of water  
138 collected at mid-depth of the water column. From each sample, 500mL were filtered on 0.2µm polycarbonate  
139 GTTP filters for microbiome analyses, 15 mL were fixed with formaldehyde for flow cytometry analysis (final

140 concentration 2%) and the remaining amount was filtered on 47mm GF/F filters (Whatman) for nutrient  
141 concentration measurement.

142 Sediment samples consisted of 5mL cryotubes. Eleven samples were collected at D0 in the bucket containing  
143 the sediment used to cover the bottom of the tanks. Five samples were collected per tank at D8: two under the  
144 open shelters (one under each), one in the closed shelter, and two randomly in other locations within the tank  
145 (hereafter, sediment samples).

146 Rocks covered with turf were rinsed with deionized water to remove interstitial seawater and rubbed with a  
147 sterile buccal swab. Twelve rocks were swabbed at D0 and three per tank at D8.

148 At D0, pieces from six individuals of each of the four macroalgae species were rinsed with deionized water to  
149 remove interstitial seawater and rubbed with a sterile buccal swab. In addition, we collected four samples per  
150 species at D0 and two per tank at D8 for analyses of nutrient content.

151 After capture at D8, the twelve fish were immediately euthanized, measured (mm), weighted (g) and dissected  
152 using tools cleaned with 70° ethanol. The last third of the gut before the cloaca, which corresponded to the  
153 hindgut, was squeezed out on a piece of parafilm paper using a sterile pipette, homogenized and frozen in a 3  
154 mL cryotube. The content of the cloaca corresponding to fish feces was stored in a cryotube at -20°C.

155 Filters, cryotubes and swabs were stored at -80°C until DNA extraction.

156

## 157 **II.2. Nutrient content in seawater and macroalgae**

158 The concentration of dissolved inorganic nutrient (NO<sub>2</sub>-NO<sub>3</sub>, NH<sub>4</sub> and PO<sub>4</sub>) was measured in the GF/F filtered  
159 seawater samples using segmented flow automatic analyzers with colorimetric detection for NO<sub>2</sub>-NO<sub>3</sub> and PO<sub>4</sub>  
160 and fluorometric detection for NH<sub>4</sub> (LER lab, Sète, France following Aminot & Kérouel, 2007 protocols).

161 The macroalgae were freeze-dried and grounded with mortar and pestle before being sent for elementary  
162 analysis of their total N and P contents using CHN analyzer (LAP lab, Montpellier, France) and ICP-AES  
163 analyzer after mineralization (LIENSs lab, La Rochelle, France), respectively.

164

## 165 **II.3. Nutrient fluxes from fishes**

### 166 *II.3.1- Estimation of nutrient excretion rates of S. rivulatus*

167 The amount of dissolved nitrogen and phosphorus excreted by *S. rivulatus* individuals was estimated using 17  
168 individuals from the acclimatization tanks (i.e. different than the one used for the main experiment but with  
169 similar body mass of 37 g on average) following protocol from Whiles et al. (2009). Fish were placed in  
170 individual plastic bags filled with 1.5L of water from the ILOR circulation system filtered on 0.2 µm.  
171 Individuals were removed from the bag after 52±23 min, on average, and the water was immediately filtered  
172 on 47mm GF/F filters and frozen. These fish were subsequently dissected and all but two individuals had their  
173 gut full of digested algae, thus our excretion measurements correspond to those of fed fish. These excretion  
174 samples were analyzed with the same protocols as for water nutrient concentration (see below) to obtain per

175 capita NH<sub>4</sub> and PO<sub>4</sub> excretion rates (*i.e.* μmol.h<sup>-1</sup>). These rates were used to estimate the flux of nutrients  
176 excreted by three *S. rivulatus* on a daily basis (*i.e.* average per capita and per hour excretion rate x 3 fish x 24  
177 hours).

178

### 179 *II.3.2- Estimation of nutrient egestion by S. rivulatus*

180 Gut volume was estimated using guts pictures from 10 individuals sampled in the wild on the shores of the  
181 Levantine basin, with a similar standard length as the experimental fish (*i.e.* 109 to 132 mm) and whose guts  
182 were full of food. The average gut diameter was obtained using ten equidistant measurements performed using  
183 imageJ and the volume was calculated using the formula for cylinders volume. Feces density was estimated  
184 by measuring the dry mass of a known volume of feces on 30 replicates. We estimated daily per capita egestion  
185 rate according to estimated gut volume and feces density and the conservative assumption that each individual  
186 fills and empties its gut only once a day. Fish feces samples were freeze-dried and ground with mortar and  
187 pestle, and their total N and P content were analyzed as for macroalgae.

188

189

## 190 **II.3. Characterization of microbial communities**

### 191 *II.3.1- Abundance of planktonic micro-organisms*

192 The abundance of prokaryotic cells was determined from a 0.5 ml sub-sample from the 15 mL formaldehyde-  
193 fixed water sample using flow cytometry (FCM) according to Marie et al. (1997). Bacteria and Archaeobacteria  
194 cannot be discriminated using this method but as the contribution of archaea in the eastern Mediterranean  
195 seawater is low and varies from 1.4% (phylum Euryarchaeota) to 12% (phylum Crenarchaeota) (De Corte et  
196 al 2009), we hereafter use FCM outputs as proxy of bacterial abundance. Subsamples of 0.5 ml were first  
197 incubated with 0.5 μl of SYBR®Green I (Molecular Probes) for 15 min at room temperature in the dark. Then  
198 the cells were enumerated using a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15 mW,  
199 488 nm, air-cooled argon laser and a standard filter set-up. True count beads (Becton Dickinson) were added  
200 to each sample as a standard. Phytoplankton biomass was estimated as chlorophyll a concentration measured  
201 by spectrofluorimetry (LS 50B Perkin Elmer) after pigment extraction in acetone 90% (MARBEC lab,  
202 Montpellier, France following Neveux & Lantoine, 1993).

203

### 204 *II.3.2- DNA extraction from microbial samples*

205 DNA extractions were performed in the molecular biology platforms of the MARBEC laboratory (UMR 9190,  
206 [www.umr-marbec.fr](http://www.umr-marbec.fr)) and at the Genseq platform ([genseq.umontpellier.fr](http://genseq.umontpellier.fr)), using the Qiagen MagAttract  
207 PowerSoil DNA KF Kit, selected for its compliance with the Earth Microbiome Project (Marotz et al., 2017).  
208 Extractions were performed in 96 well plates in which 3 wells were left empty to serve as negative controls  
209 and 3 wells were loaded using standard mock communities (ZymoBIOMICS Microbial Community DNA

210 Standards II, Zymo Research). These standards of known composition were used to evaluate the quality of our  
211 sample processing pipeline. Extraction wells were loaded using half of a GTTP filter for water samples, half  
212 of a swab for turf samples, and ~ 0.25 g of gut content and sediment samples. DNA extraction protocol included  
213 a bead beating step and a chemical lysis. DNA recovery was based on magnetic beads and automated with a  
214 Kingfisher Flex robot. DNA was eluted in 100µL of elution buffer before quantification of DNA quantity and  
215 quality using a Nanodrop 8000 spectrometer. Extracted DNA was stored at 4°C until PCR amplification, which  
216 was done the next day.

217

### 218 *II.3.3- PCR amplification*

219 PCR amplification was done using universal bacterial primers selected for their compliance with the Earth  
220 Microbiome Project (Parada et al., 2016): 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-  
221 CCGYCAATTYMTTTRAGTTT). The targeted sequence was 411 bp and corresponded to the V3-V4  
222 regions of the prokaryotic bacterial 16S rRNA gene. PCR amplification was carried out in 96 well plates in  
223 triplicate for each DNA extract and was done in a 25 µL reaction volume. The PCR mix consisted of 9.75µL  
224 of water, 0.75µL of DMSO, 0.5 µL of each primer (concentration), 12.5 µL of Phusion ready-to-use Taq mix  
225 (Phusion High-Fidelity PCR Master Mix with GC Buffer) and 1µL of DNA. After an initial denaturation of  
226 30 sec at 98°C, the PCR cycle consisted of 30 cycles of 10 sec denaturation at 98°C, 1 min annealing at 58°C  
227 and 1 min 30 sec of extension at 72°C. Final extension was held for 10 min at 72°C before keeping the  
228 reaction at 4°C. The success of PCR amplification was checked using GelRed<sup>TM</sup> on 2% agarose gel in TAE  
229 buffer and using a 100bp DNA ladder. The wells left empty during DNA extraction served as negative  
230 controls for contamination of the PCR reactions. PCR triplicates were pooled and stored at -20°C before  
231 sequencing. An amplicon library was constructed by the Genotoul platform ([www.get.genotoul.fr](http://www.get.genotoul.fr)) and  
232 sequencing was carried out using an Illumina MiSeq (2 × 250 bp) sequencer.

233

### 234 *II.3.4- Amplicon sequencing and sequences processing*

235 Reads from NGS were processed with the R software environment using the package *dada2* (Callahan et al.,  
236 2016). Briefly, the quality of the reads for each sample was inspected using graphic representations of their  
237 quality scores and reads were filtered based on their length and quality. Amplicon sequence variants (ASVs)  
238 were inferred using the *dada* algorithm (Divisive Amplicon Denoising Algorithm) after pooling dereplicated  
239 reads from all samples. Then, forward and reverse reads were merged and chimeric sequences were removed.  
240 After these steps, a third of the original sequences were kept for further analyses, on average. The taxonomic  
241 classification of ASVs was performed with the naive Bayesian RDP classifier implemented in *dada2* and using  
242 the SILVA reference database nr\_V132 (10.5281/zenodo.1172783). The quality of taxonomic assignment was  
243 assessed by bootstrap and ASVs with an assignment bootstrap value < 90 were discarded. As many ASVs were  
244 not affiliated at the genus level, ASVs sequences were blasted against the NCBI 16S rRNA bacterial database,

245 and the best hit was used to correct ASV taxonomy. We used percentage of similarity with the best hit sequence  
246 at 97% and 95% for species and genus level assignment, respectively. Otherwise the genus and species were  
247 left unassigned.

248 Several data cleaning steps were performed to remove poorly characterized and rare ASVs. First, the mock  
249 communities and blank samples were used to identify contamination from the reagents (*e.g.* extraction kit,  
250 polymerase), and these ASVs were removed from the data set (*e.g.* *Ralstonia*, *Rhizobium*). Second, ASVs not  
251 assigned to the bacterial domain, unclassified at the Family level or above, or assigned to chloroplasts and  
252 mitochondria were filtered out. Third, ASVs present in less than 2 samples were removed. ASV sequences  
253 were aligned using *mafft* implemented in *Qiime2* before being inserted in the Greengene reference  
254 phylogenetic tree (Janssen et al., 2018). The tree was then ultrametricized using *pathd8*  
255 ([www2.math.su.se/PATHd8](http://www2.math.su.se/PATHd8)).

256 We focused our analyses on the core microbiome from each of the 5 compartments of the mesocosms  
257 (planktonic, sediment, turf, macroalgae and fish gut), that is on the most frequent and/or abundant ASVs of the  
258 bacterial communities. For that, we applied a core identification algorithm (Magurran and Henderson, 2003)  
259 that uses both ASVs occurrence and abundance across communities. This method is based on the comparison  
260 of the observed abundance-occurrence distribution of ASVs with a random distribution under a stochastic  
261 Poisson model. The core ASVs identification algorithm was run on datasets from each compartment separately  
262 and then ASVs that were identified as core in at least one compartment were kept for further analyses. The  
263 final dataset was composed of 1,839 ASVs identified as core in at least one of the 5 compartments.

264

#### 265 **II.4. Statistical analyses**

266 The diversity of bacterial communities was assessed using the Hill numbers framework (Chao et al., 2014), as  
267 it embeds within a single mathematical framework alpha (*i.e.* within community) and beta (*i.e.* dissimilarity  
268 between communities) diversity components, for both taxonomic and phylogenetic facets. In addition, Hill  
269 numbers indices can put more or less weight on taxa abundances. We estimated alpha diversity as taxa richness  
270 and entropy, and we estimated beta diversity as dissimilarity in composition and structure, by using presence-  
271 absence and relative abundance data, respectively. These analyses were performed at the sample level and for  
272 three different levels of bacterial taxonomic resolution: Phylum, Family and ASV.

273 To test the effect of *S. rivulatus* on nutrient concentration (N and P), abundance of bacterioplankton, biomass  
274 of phytoplankton, alpha diversity of bacterial communities, and macroalgae nutrient content (%N and %P), we  
275 used linear mixed models (*i.e.* lmm) to account for the non-independence of replicates within tanks. The fixed  
276 effect corresponded to the treatment (fish vs control) while the random nested effect corresponded to the tank  
277 effect within each treatment. Hence, lmm were written as follow: *response variable* ~ *treatment* +  
278 (*1|treatment:tank*). Lmm were fitted using the *lmer* function in the R package *lmer* (Bates et al., 2015) and the  
279 significance of the treatment effect was assessed by ANOVA (R Development Core Team, 2011).

280 Differences in composition and structure of bacterial communities between the control and the fish treatment  
281 were assessed using PERMANOVA (Anderson, 2001) implemented as the *adonis* function from the R package  
282 *vegan* (Oksanen et al., 2016). We took into account the nested structure of our experimental design by  
283 constraining the permutations of the PERMANOVA within strata corresponding to experimental tanks. Then,  
284 for the compartments that exhibited significant differences using PERMANOVA, we used a linear discriminant  
285 approach coupled with effect size estimation (LefSe; Segata et al., 2011) to identify the organisms that  
286 consistently explained the differences between the control and the fish treatment.

287

288

### 289 **III. RESULTS**

290

#### 291 **III.1. Grazing behavior and nutrient recycling by *S. rivulatus***

292 During the experiment, the fish consumed mostly one type of macroalgae, *Ulva enteromorpha*, as suggested  
293 by the decrease in biomass from day to day and the fish gut content (we found also evidence of *Halopteris*  
294 *scoparia* consumption in the gut of one fish). Nonetheless, fish biomass decreased during the experiment in  
295 all the tanks and we noticed that fish from tank F1 did not start eating before D5 (Figure S2). Furthermore,  
296 fish from this tank lost a much higher proportion of their biomass compared to the other tanks (*i.e.* -13.6% for  
297 F1 vs -7.6, -5.2 and -2.9% for F3, F5 and F7, respectively, Table S1). Altogether, this suggested that fish from  
298 the tank F1 had a fasting behavior for several consecutive days that is different from the intense grazing  
299 behavior of *Siganus* in the wild during spring. For this reason, the data from tank F1 were not included in the  
300 analyses presented in the main text and we provided analyses with the full dataset in supplementary material  
301 (Figures S5, S6, S7).

302 The average daily flux of nutrients excreted by three *S. rivulatus* individuals (~ 100g in total) was  $1410 \pm 599$   
303  $\mu\text{mol}\cdot\text{day}^{-1}$  of  $\text{NH}_4$  and  $14 \pm 14$   $\mu\text{mol}\cdot\text{day}^{-1}$  of  $\text{PO}_4$ , corresponding to a molar N:P ratio of  $129 \pm 62$ .

304 The estimated gut volume of ~30g *S. rivulatus* individuals ( $122 \pm 7$  mm in SL) was on average of  $6.5 \pm 2.7$  mL  
305 and the density of dry feces was of  $52 \pm 40$   $\text{mg}\cdot\text{mL}^{-1}$ . The estimated daily amount of feces egested by one 30g  
306 individual is  $333$   $\text{mg}\cdot\text{day}^{-1}$ . *S. rivulatus* feces (dry mass) were composed of  $1.9 \pm 0.9$  % of N and  $0.2 \pm 0.1$  % of  
307 P, which corresponded to a N:P ratio of  $8.8 \pm 2.3$ .

308

#### 309 **III.2. Effect of *S. rivulatus* on nutrient content of water and macroalgae**

310 The presence of *S. rivulatus* had a significant positive effect on the concentration of dissolved nitrogen (Figure  
311 1, Table S2, lmm p-values < 0.05) with 144% more  $\text{NO}_3/\text{NO}_2$ , and 758% more  $\text{NH}_4$  in tanks with fish than in  
312 control tanks. Concentration of  $\text{PO}_4$  was not significantly different between the control and the treatment.

313 The presence of *S. rivulatus* had contrasting effect on the N and P content of the three remaining macroalgae  
314 species (Figure 2 and Table S3). The N content of *Padina pavonica* and *Halopteris scoparia* was significantly

315 higher in the tanks with fish compared with the control tanks (+ 35 and + 45%, respectively, lmm p-values <  
316 0.05), while no difference was found for *Treptacantha rayssiae*. By contrast, P content of the three species did  
317 not differ between the control and the fish treatment. This resulted in higher N:P values in *P. pavonica* (+ 63%)  
318 and *H. scoparia* (+ 83%) in tanks with fish.

319

### 320 **III.3 Diversity and structure of bacterial communities in different compartments of the mesocosms**

321 Bacterial communities from the gut of *S. rivulatus* hosted a much higher phylogenetic richness and diversity  
322 than other compartments of the mesocosms (*i.e.* macroalgae, sediment, turf and water, lmm, p-values < 0.05,  
323 Figure S3).

324 The fish gut microbiome had a very different composition than the macroalgae, sediment, turf and water  
325 compartments (Tables S4, S5). Planktonic and benthic bacterial communities were dominated by two phyla,  
326 Proteobacteria (23 to 48% of the sequences) and Bacteroidetes (41 to 72%) while fish microbiome contained  
327 a high proportion of Firmicutes (23%) and Verrucomicrobia (12%). Several families from the Clostridiales and  
328 Bacteroidales orders (both Firmicutes) were abundant (>10%) in fish guts but represented less than 1% of the  
329 sequences in the other compartments (Table S5). These families included the Desulfovibrionaceae (22% of  
330 sequences in fish and 98 ASVs associated with fish according to LefSE analysis), Rikenellaceae (15% and 65  
331 ASVs), Akkermansiaceae (11% and 29 ASVs), Ruminococcaceae (14% and 96 ASVs), Marinilabiliaceae (11%  
332 and 23 ASVs), Marinifilaceae (12% and 29 ASVs), Lachnospiraceae (4% and 15 ASVs) and  
333 Erysipelotrichaceae (2% and 14 ASVs). Flavobacteriaceae were the most abundant family in the macroalgae,  
334 sediment, turf and water compartments (36 to 66%) but were scarce in the fish gut (1%).

335

### 336 **III.4. Effect of *S. rivulatus* on planktonic and benthic microbial communities**

337 The biomass of phytoplanktonic micro-organisms was significantly higher in the fish tanks (Figure 3, Table  
338 S6, lmm p-values < 0.05), with concentration of chlorophyll *a* 87% higher in tanks with fish. Total bacterial  
339 abundance was also significantly higher in the fish tanks (lmm p-value < 0.05), with a 147% increase compared  
340 to control tanks.

341 *S. rivulatus* did not affect the alpha diversity of the bacterial communities in water, sediment or turf (Figure 4,  
342 Table S7, lmm p-values > 0.05). However, the presence of *S. rivulatus* significantly increased the alpha  
343 diversity of the benthic bacteria in the open shelters (lmm p-values < 0.05, for taxonomic and phylogenetic  
344 richness and taxonomic diversity) but not in the closed shelter.

345 The presence of *S. rivulatus* influenced the composition and structure of the bacterioplankton (PERMANOVA,  
346 p-value < 0.05, Figure 4), for both taxonomic and phylogenetic facets (Table S7). *S. rivulatus* significantly  
347 influenced the structure of the bacterial communities from the sediment outside of the shelters for both  
348 taxonomic and phylogenetic facets, but only at low taxonomic resolution (*i.e.* ASV and family, PERMANOVA,  
349 p-value < 0.05). Fish modified significantly the bacterial communities from the sand in open shelters at all

350 levels of taxonomic resolution, but did not influenced bacterial communities from the closed shelters or the  
351 turf (p-value > 0.05).

352

353 Among the 71 ASVs that differentiated water communities between tanks without fish and tanks with fish,  
354 48% (n = 34) were more abundant in the fish tanks (Figure 4). These mostly corresponded to Bacteroidetes  
355 from the Flavobacteriaceae (18 ASVs) family, with the two species *Polaribacter marinaquae* (9) and *Tamlana*  
356 *sedimentorum* (4) that were well represented, to Alphaproteobacteria from the Rhodobacteraceae (14), with  
357 notably the species *Marivita litorea* (12 ASVs), along with Gammaproteobacteria (2). Among the 54 ASVs  
358 that differentiated the open shelters between the control and the fish treatment, more than two thirds (70%, n  
359 = 38) were more abundant in the fish tanks. These corresponded mostly to Bacteroidetes from the  
360 Flavobacteriaceae (31), and especially from the genera *Algibacter* (10), *Polaribacter* (11) and *Actibacter* (2),  
361 but also from the Saprospiraceae (2).

362

363 The number of ASVs shared between fish gut microbiomes and the sediment under the open shelters increased  
364 from 106 ASVs in the control to 137 in the fish tanks (+ 29%). These shared ASVs represented 13 and 22% of  
365 the sequences of the open shelter communities from the control and the fish treatment, respectively.  
366 Interestingly, among the ASVs shared between compartments and only observed in the fish tanks, we found a  
367 majority of bacterial families identified as characteristic of the fish gut microbiome such as the  
368 Ruminococcaceae (18 ASVs, Firmicutes), Akkermansiaceae (6, Verrucomicrobia), Rikenellaceae (15,  
369 Bacteroidetes) and Desulfovibrionaceae (15, Deltaproteobacteria). Similarly, the number of ASVs shared  
370 between fish gut microbiome and bacterioplankton increased from the control (40) to the fish tanks (75, +  
371 88%), but the total abundance of these ASVs did not differ between the control and the fish treatment. Again,  
372 we observed that this supplementary ASV in the fish tanks corresponded to bacterial families abundant in the  
373 fish microbiome (6 Rikenellaceae ASVs, 14 Desulfovibrionaceae and 4 Ruminococcaceae).

374

375

## 376 **IV. DISCUSSION**

377

### 378 **IV.1. Nutrient cycling by *Siganus rivulatus***

379 *S. rivulatus* excreted dissolved nutrients with a molar N:P ratio of 129±62, much higher than the Redfield ratio  
380 (16:1) and the initial N:P ratio in the water at the start of the experiment (29±9). The N:P ratio of nutrients  
381 excreted by *S. rivulatus* appears particularly high compared with those obtained in similar conditions from  
382 native non-herbivorous Mediterranean fishes (Villéger et al., 2012), for which N:P ranges from 17 to 60. Daily  
383 amounts of dissolved nitrogen released by three fish (30 grams each) is 4.6 times higher than the standing  
384 stocks of NH<sub>4</sub> (1.76 μmol.L<sup>-1</sup>) at the beginning of the experiment (0.38 μmol.L<sup>-1</sup> of NH<sub>4</sub>) in the 800L tanks.

385 Excretion of dissolved phosphorus by the three fish ( $0.05 \mu\text{mol.L}^{-1}$  of  $\text{PO}_4$ ) was of the same order of magnitude  
386 (0.4 times) than initial  $\text{PO}_4$  concentration ( $0.02 \mu\text{mol.L}^{-1}$ ).

387 In our experiment, *S. rivulatus* consumed almost exclusively one out of the four species of macroalgae that  
388 were provided as food sources (*i.e.* *Ulva enteromorpha*), but in the wild this species has been reported to feed  
389 preferentially on epilithic algal matrix (EAM, termed turf in this study, Vergés et al., 2014a). In our experiment,  
390 feces of *S. rivulatus* contained  $1.9 \pm 0.8$  %N,  $0.2 \pm 0.1$  %P had a N:P of  $8.8 \pm 2.3$  N:P. The 3 individuals hence  
391 released daily in each tank at least 19 mg of N and 2.3 mg of P through defecation and 19.7 mg of N and 0.4  
392 mg of P through excretion. Consequently, *S. rivulatus* released as much N through excretion of dissolved  
393 inorganic forms through egestion of particulate organic forms, while it released 5 times more P as particulate  
394 organic forms than dissolved inorganic forms.

395

#### 396 **IV.2. Indirect effect of *S. rivulatus* on planktonic microbes through the excretion of dissolved nutrients**

397 Presence of *S. rivulatus* resulted in an increase of the biomass of phytoplanktonic (+87%) and abundance of  
398 procaryotic (+147%) cells, as observed elsewhere using juvenile fasting fish (Villéger et al., 2019). Despite  
399 this increase in abundance of planktonic microbes, dissolved nitrogen was still much higher in the fish tanks  
400 than in the controls, suggesting an excess of N in the system in the presence of the fish. In contrast,  $\text{PO}_4$   
401 concentrations were similar in the control and the fish treatment, and did not change during the experiment,  
402 suggesting that P was the limiting nutrient. The higher phytoplanktonic biomass is hence likely the result of an  
403 indirect bottom-up effect of the dissolved nitrogen and phosphorus excreted by the fish.

404 As *U. enteromorpha* was completely grazed in the fish tanks, it is likely that the increase in nitrogen content  
405 (N% and N:P) in the tissues of two species of brown macroalgae (*Halopteris scoparia* and *Padina pavonica*)  
406 was due to the higher nutrient concentration in the water that resulted from the recycling of *U. enteromorpha*  
407 nutrients by *S. rivulatus*, as observed with other herbivorous fish and macroalgae in coral reef ecosystems  
408 (Burkepile et al., 2013).

409 One bacterial family from the water column responded particularly strongly to the presence of the fish, the  
410 Flavobacteriaceae, with two genera increasing in abundance in the fish tanks (*Polaribacter*, *Tamlana*). These  
411 are gram negative, aerobic and non-motile heterotrophic bacteria commonly observed in marine coastal  
412 environments (in water, on particles and in invertebrate microbiomes) and they likely benefited from the  
413 enhanced pool of particulate organic matter originating from dead phytoplanktonic cells, degraded macroalgae,  
414 dissolved feces, and dead cells flaking off the fish. Another family stood out, the Rhodobacteraceae  
415 (Alphaproteobacteria), with notably the genus *Marivita* containing photosynthesis-related and antimicrobial  
416 genes. Several *Marivita* sp. were isolated from marine phytoplankton cultures suggesting their involvement in  
417 mutualistic interactions with eukaryotes (e.g. Hwang et al., 2009; Zhou et al., 2021). Their development in the  
418 presence of *S. rivulatus* would therefore be an indirect effect of fish excretion via the growth of phytoplankton.

419 We also found that the number of ASVs shared between the fish gut and the water communities increased with  
420 the presence of the fish (40 to 75). These additional common ASVs corresponded mostly to families associated  
421 with *S. rivulatus* gut microbiome (Rikenellaceae, Desulfovibrionaceae and Ruminococcaceae), which  
422 suggests they come from fish feces. However, they represented < 2% of sequences in the water column,  
423 suggesting the planktonic way of life not being favorable to them. Nonetheless, these microorganisms were  
424 detectable only in the water from tanks with fish, supporting the hypothesis that the transit through the gut of  
425 macroorganisms participate in the maintenance of populations of rare planktonic microbes and thus increases,  
426 at least temporarily, the likelihood for another potential host to enter in contact with these microbes  
427 (Troussellier et al., 2017). Further investigations are thus needed to determine the fate of bacteria present in  
428 fish feces and whether planktonic bacteria benefit directly from nutrients excreted by fish or indirectly from  
429 the compounds release by the phytoplankton (Fouilland et al., 2014).

430

#### 431 **IV.3. Direct effect of *S. rivulatus* on benthic microbes through localized defecation**

432 The presence of fecal pellets on sediment in shelters at the end of the experiment is consistent with observation  
433 of *Siganus spp.* in the wild which sleep very close to the sediment, often leaning on rocks or in crevices in  
434 small groups (2-5 individuals). In addition, most the individuals caught at night (10-11 PM) in the nearby  
435 ecosystems during other samplings had their gut full of algae material, while the ones caught in the morning  
436 had empty guts, suggesting that an individual defecates each night several grams of feces.

437 The effect of fish on sediment microbial communities, and particularly those located under the shelters, was  
438 stronger than their effect on planktonic microbes. Indeed, bacterial communities under the shelters showed an  
439 increased taxonomic and phylogenetic diversity and shared a higher proportion of ASVs with the fish gut  
440 microbiome (106 to 137 in control and fish tanks, respectively). These shared ASVs originated from taxa  
441 characteristic of *S. rivulatus* gut microbiome (Ruminococcaceae, Akkermansiaceae, Rikenellaceae and  
442 Desulfovibrionaceae) which represented a higher proportion of the sequences in the sediment from the fish  
443 tanks (13 vs. 22%), and even more when compared with the water communities (< 2%). This suggests that  
444 feces released under the shelters modified directly the composition of the sediment microbial communities and  
445 explain the gain of phylogenetic diversity due to the appearance of several ASVs from the Firmicutes  
446 (including Ruminococcaceae), a phylum not observed in other sediment samples. The benthic taxa responding  
447 the most to the presence of the fish were from the Flavobacteriaceae family (as in the water), with notably two  
448 genera that differentiated the control and the fish treatment (*Actibacter* and *Algibacter*). These facultative-  
449 anaerobic heterotrophic and gliding bacteria have been isolated from the surfaces of macroalgae (Hyun et al.,  
450 2015; Martin et al., 2015; Nedashkovskaya et al., 2004), and thus were ingested during grazing (as they  
451 represented 0.8 to 14% of sequences in *U. intestinalis* microbiome), transited through the fish gut where they  
452 contributed to the food degradation and increased in abundance before being egested as feces. Two bacterial  
453 families, the Saprospiraceae and Sphingomonadaceae, that were found in higher abundance under the shelters

454 in the fish tanks are characteristic of macroalgae microbiome (Figure 3), supporting further the local  
455 enrichment of sediment microbes via feces egestion.

456

## 457 **V. CONCLUSION**

458 In this study, we used a mesocosm experiment to test the effect of an invasive marine herbivorous fish (*Siganus*  
459 *rivulatus*) on planktonic and benthic microbial communities. We found that *S. rivulatus* affected planktonic  
460 microbes through the indirect bottom-up effect induced by excreted nutrients while it affected benthic microbes  
461 due to release of nutrients in its feces and microbes from its gut microbiome. Further studies are needed to test  
462 whether the increases of phytoplanktonic biomass and of their associated bacterial consortia (*i.e.* phycosphere),  
463 result in enhanced nutrient turnover when this biomass is degraded by heterotrophic prokaryotes, and could  
464 eventually favor eutrophication of the water column. Similarly, future studies should assess how the increase  
465 in organic matter content and in the microbial load of the surface sediment in the fish defecation hotspots,  
466 affect the oxygen concentration in the sediment and hence the biogeochemical processes supported by benthic  
467 bacteria.

468 This experiment was designed to mimic the natural ecosystems of the South-Eastern Mediterranean Sea where  
469 *Siganus spp.* have replaced natives herbivores (Rilov et al., 2018; Frid et al., 2022). However, there are still  
470 many areas of the Mediterranean Sea (e.g. Tunisia coast, islands in Ionian and Aegean Seas) where *Siganus*  
471 co-occur with the native Mediterranean herbivores (*Sarpa salpa* and *Sparisoma cretense*). Therefore, to have  
472 a more comprehensive view of the consequence of the *Siganus* invasion, we need to assess the impacts of these  
473 native herbivorous fishes on microbes as they could have different food assimilation efficiency or metabolic  
474 rates and different nocturnal behavior. More generally, a better understanding of the functioning of coastal  
475 ecosystems facing global changes requires assessments of excretion and egestion rates by fishes from all  
476 trophic levels.

477

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487

## 488 **Data, code and materials**

489 Raw sequence data and associated metadata were deposited on DataDryad, under the accession number  
490 <https://datadryad.org/stash/dataset/doi:10.5061/dryad.2280gb5r7>

491  
492 Processed sequence data, other data supporting this manuscript and the code used for analyses are available  
493 on github: [https://github.com/sebastienvilleger/exofishmed\\_mesocosm\\_experiment](https://github.com/sebastienvilleger/exofishmed_mesocosm_experiment)

494

495

496

#### 497 **Competing interests**

498

499 The authors declare that they are not competing any interests relative to this work.

500

501

#### 502 **Authors' contributions**

503

504 AE and SV designed the experiment. AE, AA, DSG and SV performed the experiment and collected  
505 the samples. AA, TB, FR, ARM and FF processed the samples. AE and VC analyzed the data. GR  
506 and JB provided the fish and the experimental facilities. AE wrote the first draft of the paper and all  
507 authors contributed to revisions.

508

509

510

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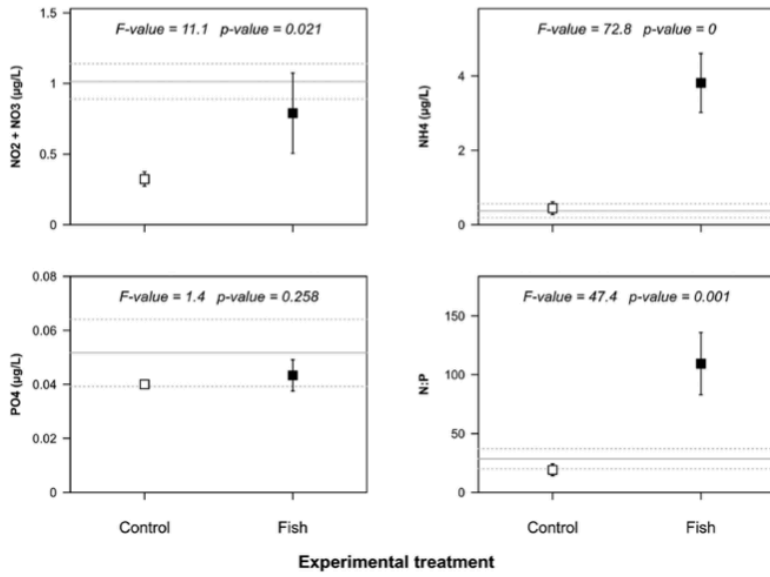
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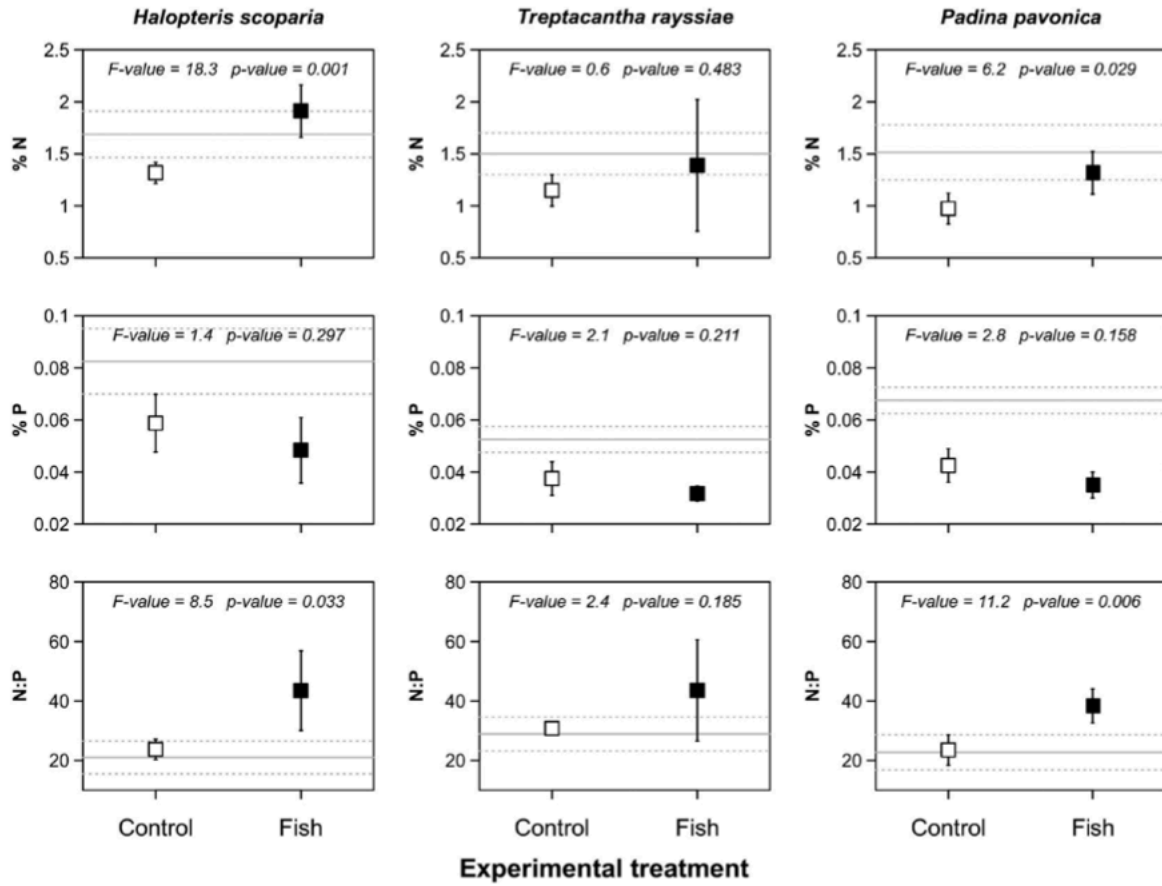
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667 **Figure 1. Effect of *Siganus rivulatus* on nutrient concentration in the water.**  
 668 Squares and error bars represent mean  $\pm$  SD across tanks. The gray and dashed lines represent mean  
 669  $\pm$  SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.  
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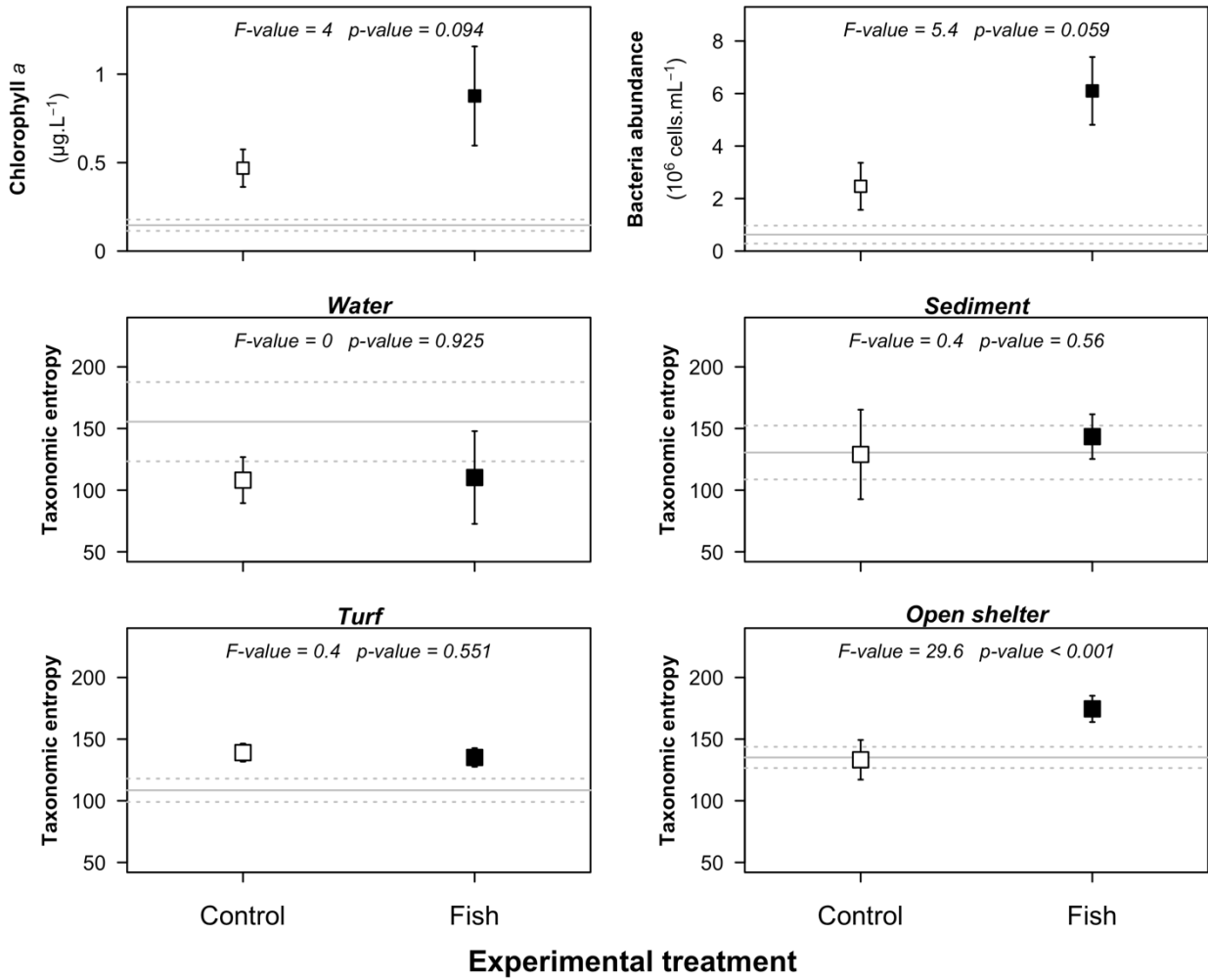
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675 **Figure 2. Nutrients content of macroalgae at the end of the experiment.**  
 676 Squares and error bars represent mean  $\pm$  SD across tanks. The gray and dashed lines represent mean  
 677  $\pm$  SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.  
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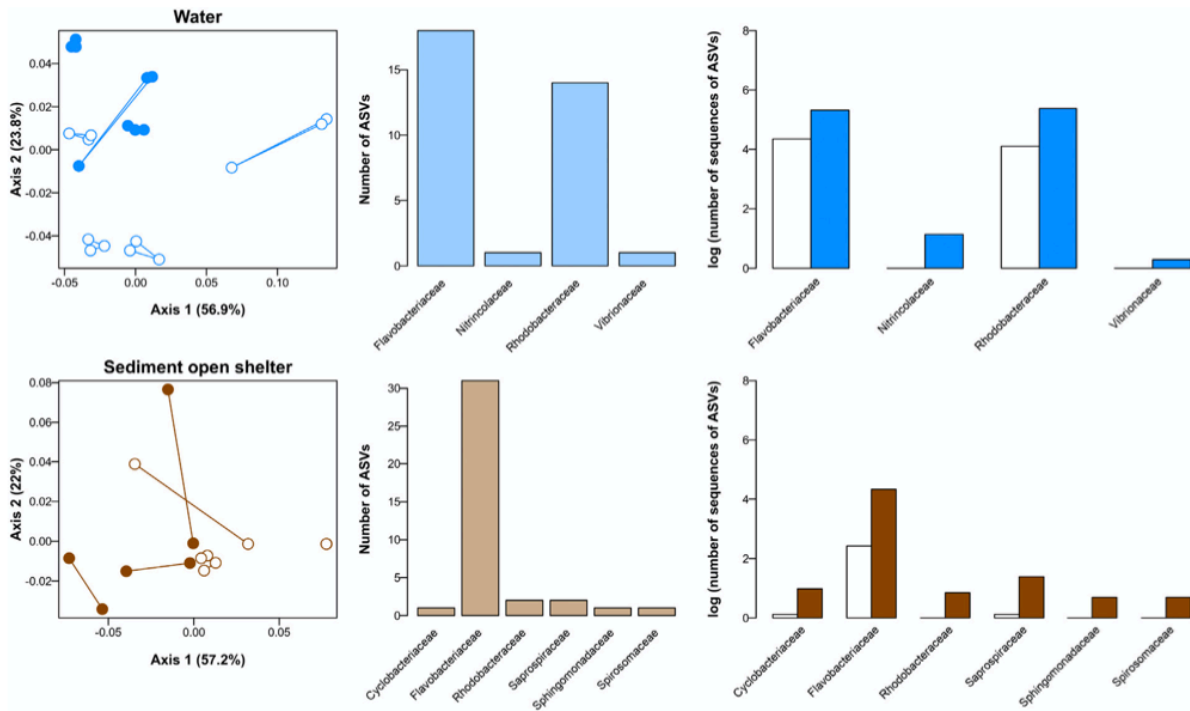
681 **Figure 3. Effect of *Siganus rivulatus* on the abundance of planktonic microbes and the**  
 682 **taxonomic entropy of communities from different compartments of the mesocosms.**  
 683 Squares and error bars represent mean  $\pm$  SD across tanks. The gray and dashed lines represent mean  
 684  $\pm$  SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.  
 685



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687 **Figure 4. Effect of *Siganus rivulatus* on the phylogenetic structure of planktonic and benthic**  
 688 **microbial communities.**

689 The plots in the left columns represent PCoA ordination of phylogenetic abundance-weighted  
 690 dissimilarity between samples from the control (open circles) and fish (black circles) treatments  
 691 (samples from the same tank are linked together). Plots from the middle column represent, for the  
 692 most common bacterial families, the number of ASVs that differentiated the control and fish  
 693 treatment, according to LefSE analysis, and that were more abundant in the fish tanks. Plots from  
 694 the right column represent for each family the logged summed abundance of the ASVs identified by  
 695 LefSE in the control (white bars) and fish (black bars) treatments.  
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