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

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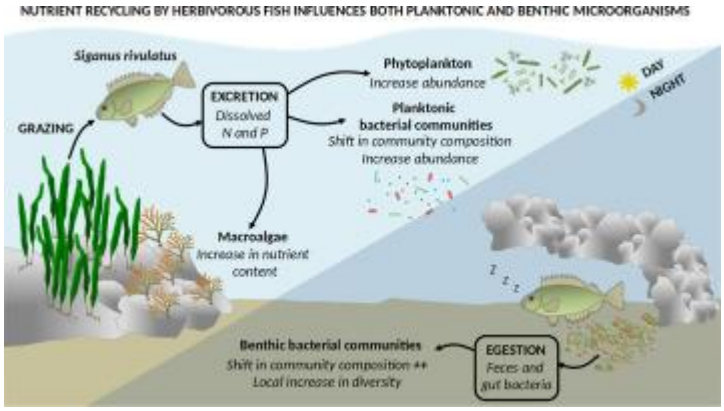
Abstract :

Direct and indirect impacts by invasive animals on plants and other animals through predation and competition have been evidenced in many ecosystems. For instance, the rabbitfish Siganus rivulatus, originating from the Red Sea, is now the most abundant species in costal habitats of South-Eastern Mediterranean Sea where it overgrazes algae. However, little is known about its impacts on microbes through release of metabolic wastes and feces. We used a mesocosm experiment to test the effect of S. rivulatus on planktonic and benthic microbial communities. Excretion of dissolved nutrients by fish resulted in higher concentrations of dissolved inorganic nitrogen (NH4, NO2/NO3). This increase in availability of N was associated with higher N content in macroalgae, higher biomass of phytoplankton, higher abundance of bacterioplankton and shift in the structure of planktonic bacterial communities. The feces released mostly under the shelters where the fish rest at night, led to significant increases in diversity of sediment bacterial communities and shifts in their structure. The impact of S. rivulatus on planktonic microbes was related to the indirect bottom-up effect induced by excreted dissolved nutrients while its effect on benthic microbes was due to the direct release of both organic matter and microbes present in feces. Overall, this first evidence of the impacts of invasive species on planktonic and benthic microbes highlights that ongoing changes in fish biodiversity could have ecosystem-wide consequences.

Graphical abstract



Highlights

► A herbivorous rabbitfish from the Red Sea has invaded the Mediterranean Sea. ► Excretion of nutrients by rabbitfish increases the abundance of planktonic microbes. ► Excretion of nutrients by rabbitfish changes the diversity of planktonic bacteria. ► Excretion of nutrients by rabbitfish changes the nutrient content of macroalgae. ► Egestion of feces by rabbitfish changes the diversity of benthic bacteria.

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

I. INTRODUCTION

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

In addition, herbivorous fishes influence nutrient dynamics in aquatic ecosystems through consumer-driven nutrient dynamics (Capps & Flecker 2013; Burkepile et al 2013). Indeed, all fish excrete ammonia (NH_4^+) and phosphate (PO_4) as dissolved metabolic wastes (Vanni 2002; Ip and Chew, 2010). Nutrient fluxes produced by fish communities reach levels comparable with other biogeochemical processes such as nitrogen fixation (Meyer and Schultz, 1985; Allgeier et al., 2017). Thus, in coral reefs, the nutrient excretion by fish could increase the nutrient content of benthic organisms such as corals and macroalgae (Burkepile et al., 2013, Shantz et al 2015). In addition, nutrient excretion by carnivorous fish can increase the abundance of both planktonic microalgae and bacteria and modify their diversity by favoring some taxa (Schaus & Vanni 2000, Villéger et al., 2019). However, there is currently no assessment of the effect of nutrient excretion by herbivorous fish on multiple compartments of marine ecosystems such as macroalgae, bacterioplankton and phytoplankton.

Fishes also recycle nutrients through the egestion of feces that are rich in particulate forms of organic nitrogen and phosphorous. For instance, the feces of carnivorous fishes often have a higher nutritional value than the regular food of herbivorous fishes, and can support the existence of fecal foodwebs (Robertson, 1982). Herbivorous fishes counterbalance their nutrient poor diet by eating continuously throughout the day

and thus tend to defecate several times per day (Vermeij et al., 2013), which results in the recycling of large amounts of nutrients through feces deposition. These amounts could be especially high in refugia and sleeping areas (Bray et al., 1981), where they could stimulate benthic microbes because of their nutrient content. However, the effect of fish feces on benthic microbes has only been studied in the context of marine aquaculture (Quero et al., 2020), where fish densities exceed those observed in the natural environment. Fish can also influence environmental microbial communities as feces contain high abundance of active bacteria originating from the gut microbiome (Smriga et al., 2010; Troussellier et al., 2017. However, the influence of release of fecal microbes on the diversity and structure of environmental microbial communities remains to be tested.

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

In this study, we used an experimental approach in mesocosms to test three hypotheses: (i) the excretion of nutrients by *Siganus rivulatus* increases the nutrient content in macroalgae not grazed; (ii) the excretion of nutrients by *Siganus rivulatus* increases the abundance of planktonic bacteria and microalgae and modifies

the diversity of their communities; (iii) the egestion of feces by *S. rivulatus* modifies the diversity of benthic microbial communities, especially under their resting spots.

II. MATERIAL AND METHODS

II.1. Experimental design

II.1.1- Mesocosm setup

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

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

of the fish on the bottom sediment could be only through nutrient excreted in the water not through egestion of feces.

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

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

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

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

II.1.2- Samples collection

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

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

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

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

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

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

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

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

II.2. Nutrient content in seawater and macroalgae

The concentration of dissolved inorganic nutrient (NO_2-NO_3, NH_4 and PO_4) was measured in the GF/F filtered seawater samples using segmented flow automatic analyzers with colorimetric detection for NO_2-NO_3 and PO_4 and fluorometric detection for NH_4 (LER lab, Sète, France following Aminot & Kérouel, 2007 protocols).

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

II.3. Nutrient fluxes from fishes

II.3.1- Estimation of nutrient excretion rates of S. rivulatus

The amount of dissolved nitrogen and phosphorus excreted by *S. rivulatus* individuals was estimated using 17 individuals from the acclimatization tanks (i.e. different than the one used for the main experiment but with similar body mass of 37 g on average) following protocol from Whiles et al. (2009). Fish were placed in individual plastic bags filled with 1.5L of water from the ILOR circulation system filtered on 0.2 µm. Individuals were removed from the bag after 52±23 min, on average, and the water was immediately filtered on 47mm GF/F filters and frozen. These fish were subsequently dissected and all but two individuals had their gut full of digested algae, thus our excretion measurements correspond to those of fed fish. These excretion samples were analyzed with the same protocols as for water nutrient concentration (see below) to obtain per capita NH_4 and PO_4 excretion rates (*i.e.* $\mu mol.h^{-1}$). These rates were used to estimate the flux of nutrients excreted by three *S. rivulatus* on a daily basis (*i.e.* average per capita and per hour excretion rate x 3 fish x 24 hours).

II.3.2- Estimation of nutrient egestion by S. rivulatus

Gut volume was estimated using guts pictures from 10 individuals sampled in the wild on the shores of the Levantine basin, with a similar standard length as the experimental fish (*i.e.* 109 to 132 mm) and which guts were full of food. The average gut diameter was obtained using ten equidistant measurements performed using imageJ and the volume was calculated using the formula for cylinders volume. Feces density was estimated by measuring the dry mass of a known volume of feces on 20 replicates (from individuals sampled in the mesocosms and acclimation tanks). We estimated daily per capita egestion rate according to estimated gut volume and feces density and the conservative assumption that each individual fills and empties its gut only once a day. Fish feces samples were freeze-dried and ground with mortar and pestle, and their total N and P content were analyzed as for macroalgae.

II.3. Characterization of microbial communities

II.3.1- Abundance of planktonic micro-organisms

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

II.3.2- DNA extraction from microbial samples

DNA extractions were performed in the molecular biology platforms of the MARBEC laboratory (UMR 9190, www.umr-marbec.fr) and at the Genseq platform (genseq.umontpellier.fr), using the Qiagen MagAttract PowerSoil DNA KF Kit, selected for its compliance with the Earth Microbiome Project (Marotz et al., 2017). Extractions were performed in 96 well plates in which 3 wells were left empty to serve as negative controls and 3 wells were loaded using standard mock communities (ZymoBIOMICS Microbial Community DNA Standards II, Zymo Research). These standards of known composition were used to evaluate the quality of our sample processing pipeline. Extraction wells were loaded using half of a GTTP filter for water samples, half of a swab for turf samples, and ~ 0.25 g of gut content and sediment samples. DNA extraction protocol included a bead beating step and a chemical lysis. DNA recovery was based on magnetic beads and automated with a Kingfisher Flex robot. DNA was eluted in 100µL of elution buffer before quantification of DNA quantity and quality using a Nanodrop 8000 spectrometer. Extracted DNA was stored at 4°C until PCR amplification, which was done the next day.

II.3.3- PCR amplification

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

II.3.4- Amplicon sequencing and sequences processing

Reads from NGS were processed with the R software environment using the package *dada2* (Callahan et al., 2016). Briefly, the quality of the reads for each sample was inspected using graphic representations of their quality scores and reads were filtered based on their length and quality. Amplicon sequence variants (ASVs) were inferred using the dada algorithm (Divisive Amplicon Denoising Algorithm) after pooling dereplicated reads from all samples. Then, forward and reverse reads were merged and chimeric sequences were removed. After these steps, a third of the original sequences were kept for further analyses, on average. The taxonomic classification of ASVs was performed with the naive Bayesian RDP classifier implemented in *dada2* and using the SILVA reference database nr_V132 (10.5281/zenodo.1172783). The quality of taxonomic assignment was assessed by bootstrap and ASVs with an assignment bootstrap value < 90 were discarded. As many ASVs were not affiliated at the genus level, ASVs sequences were blasted against the NCBI 16S rRNA

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

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

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

II.4. Statistical analyses

The diversity of bacterial communities was assessed using the Hill numbers framework (Chao et al., 2014), as it embeds within a single mathematical framework alpha (*i.e.* within community) and beta (*i.e.* dissimilarity between communities) diversity components, for both taxonomic and phylogenetic facets. In addition, Hill numbers indices can put more or less weight on taxa abundances. We estimated alpha diversity as taxa richness and entropy, and we estimated beta diversity as dissimilarity in composition and structure,

by using presence-absence and relative abundance data, respectively. These analyses were performed at the sample level and for three different levels of bacterial taxonomic resolution: Phylum, Family and ASV.

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

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

III. RESULTS

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

During the experiment, the fish consumed mostly one type of macroalgae, *Ulva enteromorpha*, as suggested by the decrease in biomass from day to day and the fish gut content (we found also evidence of *Halopteris scoparia* consumption in the gut of one fish). Nonetheless, fish biomass decreased during the experiment in all the tanks and we noticed that fish from tank F1 did not start eating before D5 (Figure S2). Furthermore, fish from this tank lost a much higher proportion of their biomass compared to the other tanks (*i.e.* -13.6% for F1 *vs* -7.6, -5.2 and -2.9% for F3, F5 and F7, respectively, Table S1). Altogether, this suggested that fish

from the tank F1 had a fasting behavior for several consecutive days that is different from the intense grazing behavior of *Siganus* in the wild during spring. For this reason, the data from tank F1 were not included in the analyses presented in the main text and we provided analyses with the full dataset in supplementary material (Figures S5, S6, S7).

The average daily flux of nutrients excreted by three *S. rivulatus* individuals (~ 100g in total) was 1410±599 µmol.day^{-1} of NH_4 and 14±14 µmol.day^{-1} of PO_4, corresponding to a molar N:P ratio of 129±62.

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

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

The presence of *S. rivulatus* had a significant positive effect on the concentration of dissolved nitrogen (Figure 1, Table S2, lmm p-values < 0.05) with 144% more NO_3/NO_2, and 758% more NH_4 in tanks with fish than in control tanks. Concentration of PO_4 was not significantly different between the control and the treatment.

The presence of *S. rivulatus* had contrasting effect on the N and P content of the three remaining macroalgae species (Figure 2 and Table S3). The N content of *Padina pavonica* and *Halopteris scoparia* was significantly higher in the tanks with fish compared with the control tanks (+ 35 and + 45%, respectively, lmm p-values < 0.05), while no difference was found for *Treptacantha rayssiae*. By contrast, P content of the three species did not differ between the control and the fish treatment. This resulted in higher N:P values in *P. pavonica* (+ 63%) and *H. scoparia* (+ 83%) in tanks with fish.

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

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

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

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

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

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

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

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

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

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

IV. DISCUSSION

IV.1. Nutrient cycling by *Siganus rivulatus*

S. rivulatus excreted dissolved nutrients with a molar N:P ratio of 129±62, much higher than the Redfield ratio (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 excreted by *S. rivulatus* appears particularly high compared with those obtained in similar conditions from native non-herbivorous Mediterranean fishes (Villéger et al., 2012), for which N:P ranges from 17 to 60. Daily amounts of dissolved nitrogen released by three fish (30 grams each) is 4.6 times higher than the standing stocks of NH_4 (1.76 µmol.L^{-1}) at the beginning of the experiment (0.38 µmol.L^{-1} of NH_4) in the 800L tanks. Excretion of dissolved phosphorus by the three fish (0.05 µmol.L^{-1} of PO_4) was of the same order of magnitude (0.4 times) than initial PO_4 concentration (0.02 µmol.L^{-1}).

In our experiment, *S. rivulatus* consumed almost exclusively one out of the four species of macroalgae that were provided as food sources (*i.e. Ulva enteromorpha*), but in the wild this species has been reported to feed preferentially on epilithic algal matrix (EAM, terrace turf in this study, Vergés et al., 2014a). In our experiment, feces of *S. rivulatus* contained 1.9±0.8 %N, 0.2±0.1 %P had a N:P of 8.8±2.3 N:P. The 3 individuals hence 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 mg of P through excretion. Consequently, *S. rivulatus* released as much N through excretion of dissolved inorganic forms through egestion of particulate organic forms, while it released 5 times more P as particulate organic forms than dissolved inorganic forms.

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

Presence of *S. rivulatus* resulted in an increase of the biomass of phytoplanktonic (+87%) and abundance of procaryotic (+147%) cells, as observed elsewhere using juvenile fasting fish (Villéger et al., 2019). Despite this increase in abundance of planktonic microbes, dissolved nitrogen was still much higher in the fish tanks than in the controls, suggesting an excess of N in the system in the presence of the fish. In contrast, PO_4 concentrations were similar in the control and the fish treatment, and did not change during the experiment, suggesting that P was the limiting nutrient. The higher phytoplanktonic biomass is hence likely the result of an indirect bottom-up effect of the dissolved nitrogen and phosphorus excreted by the fish.

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

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

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

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

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

The effect of fish on sediment microbial communities, and particularly those located under the shelters, was stronger than their effect on planktonic microbes. Indeed, bacterial communities under the shelters showed an increased taxonomic and phylogenetic diversity and shared a higher proportion of ASVs with the fish gut microbiome (106 to 137 in control and fish tanks, respectively). These shared ASVs originated from taxa characteristic of *S. rivulatus* gut microbiome (Ruminococcaceae, Akkermansiaceae, Rikenellaceae and Desulfovibrionaceae) which represented a higher proportion of the sequences in the sediment from the fish tanks (13 *vs.* 22%), and even more when compared with the water communities (< 2%). This suggests that feces released under the shelters modified directly the composition of the sediment microbial communities and explain the gain of phylogenetic diversity due to the appearance of several ASVs from the Firmicutes (including Ruminoccoccaceae), a phylum not observed in other sediment samples. The benthic taxa responding the most to the presence of the fish were from the Flavobacteriaceae family (as in the water), with notably two genera that differentiated the control and the fish treatment (*Actibacter* and *Algibacter*). These facultative-anaerobic heterotrophic and gliding bacteria represented 0.8 to 14% of sequences in *U. intestinalis* microbiomes confirming they grow on the surfaces of macroalgae (Hyun et al., 2015; Martin et al., 2015; Nedashkovskaya et al., 2004). They were thus ingested during grazing, transited through the fish gut where they contributed to the food degradation and increased in abundance before being egested as feces. Two bacterial families, the Saprospiraceae and Sphingomonadaceae, that were found in higher abundance under the shelters in the fish tanks are also characteristic of macroalgae microbiome (Figure 3), supporting further the local enrichment of sediment microbes via feces egestion.

V. CONCLUSION

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

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

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Data, code and materials

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

Processed sequence data, other data supporting this manuscript and the code used for analyses are available on github: https://github.com/sebastienvilleger/exofishmed_mesocosm_experiment

Competing interests

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

Authors' contributions

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

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Figure 1: Effect of *Siganus rivulatus* on nutrient concentration in the water
Squares and error bars represent mean±SD across tanks. The gray and dashed lines represent mean±SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.

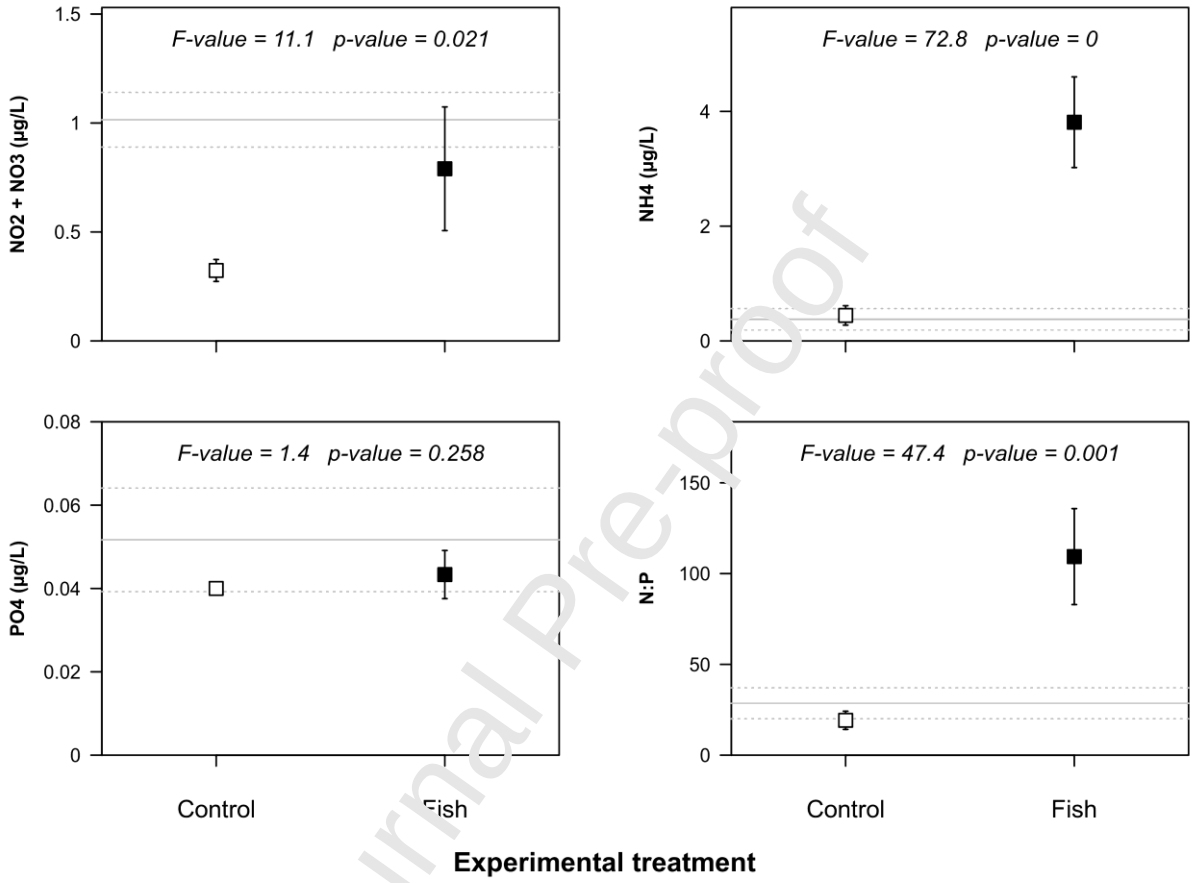


Figure 2: Nutrients content of macroalgae at the end of the experiment
Squares and error bars represent mean±SD across tanks. The gray and dashed lines represent mean±SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.

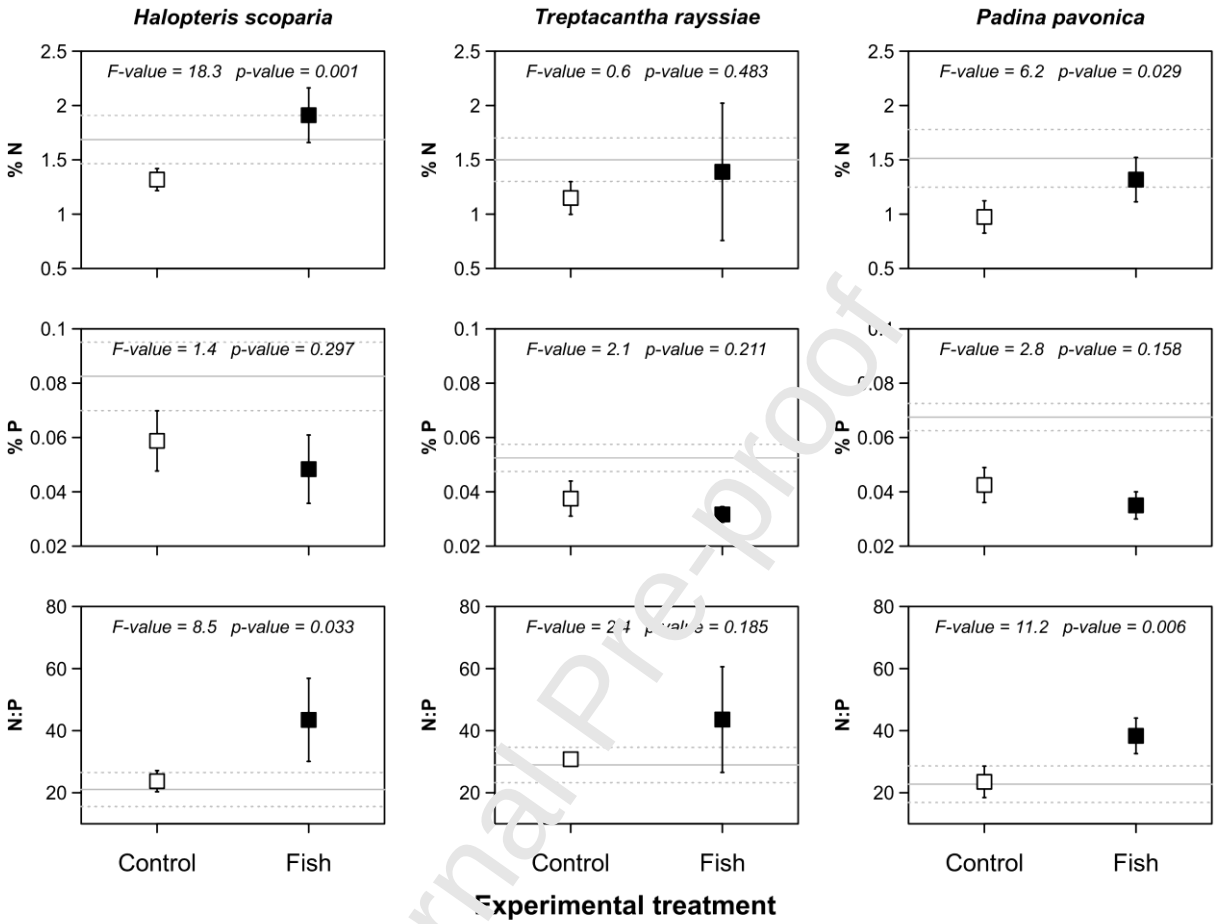


Figure 3: Effect of *Siganus rivulatus* on the abundance of planktonic microbes and the taxonomic richness of communities from different compartments of the mesocosms
Squares and error bars represent mean±SD across tanks. The gray and dashed lines represent mean±SD at D0. F and p-values correspond to fixed treatment effect in the linear mixed model.

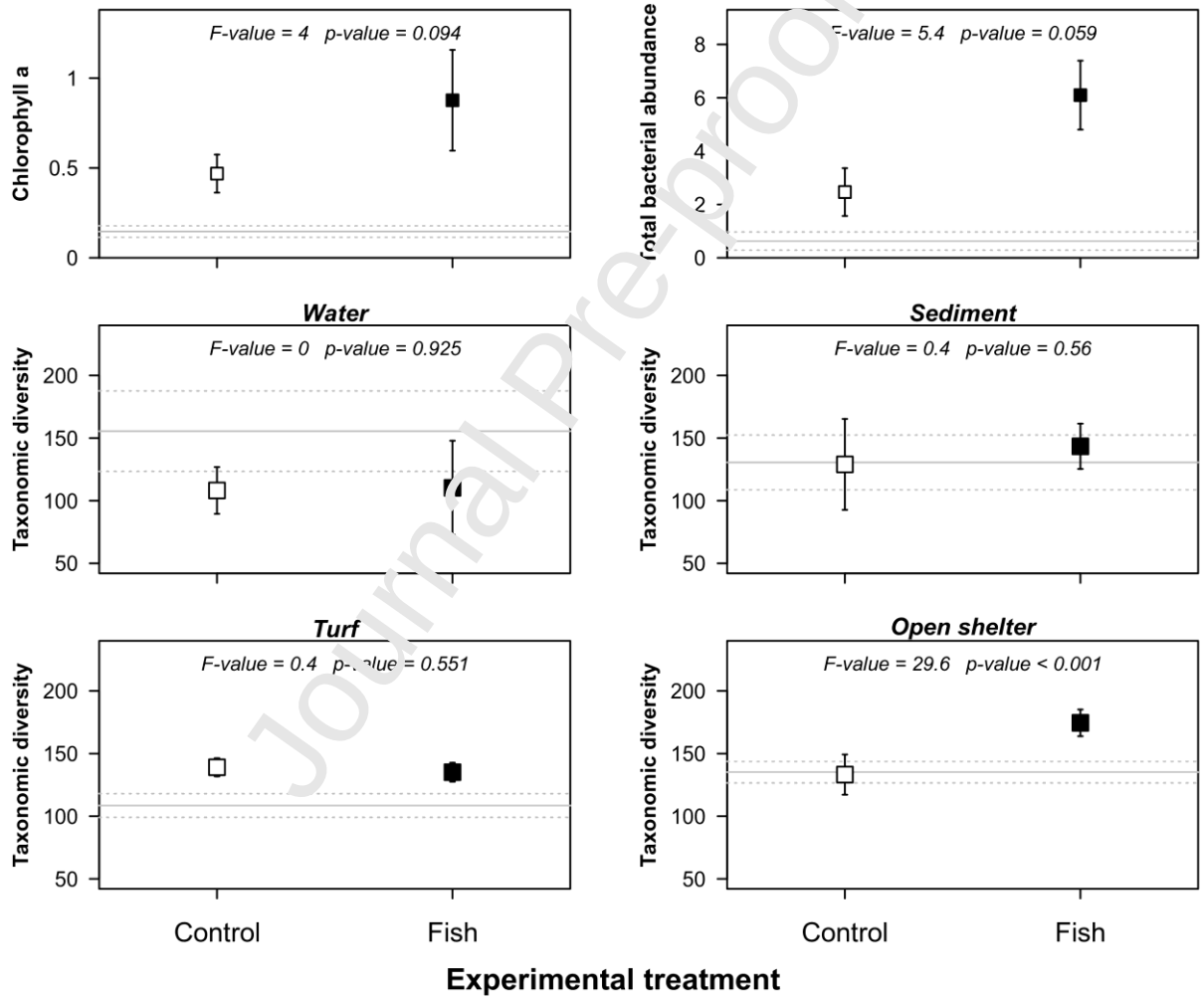
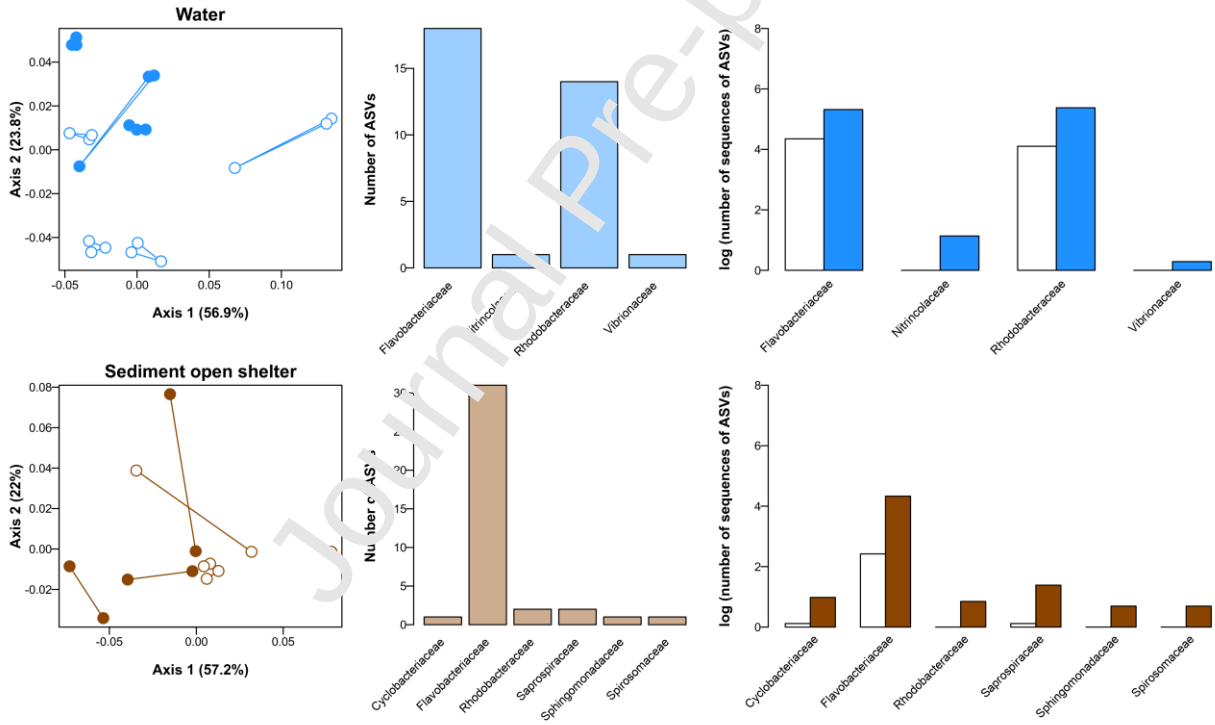
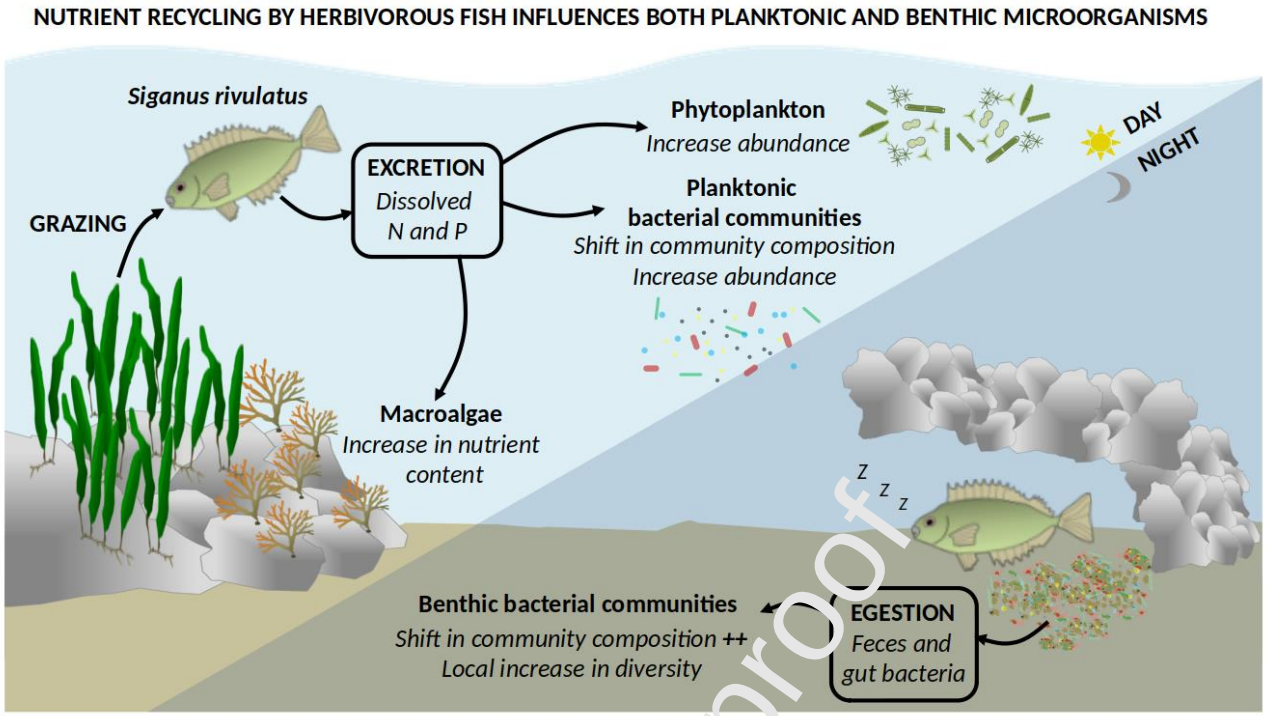


Figure 4: Effect of *Siganus rivulatus* on the phylogenetic structure of planktonic and benthic microbial communities

The plots in the left columns represent PCoA ordination of phylogenetic abundance-weighted (q = 1) dissimilarity between samples from the control (open circles) and fish (black circles) treatments (samples from the same tank are linked together). Plots from the middle column represent, for the most common bacterial families, the number of ASVs that differentiated the control and fish treatment, according to LefSE analysis, and that were more abundant in the fish tanks. Plots from the right column represent for each family the logged summed abundance of the ASVs identified by LefSE in the control (white bars) and fish (black bars) treatments



Graphical abstract



Highlights

- A herbivorous rabbitfish from the Red Sea has invaded the Mediterranean Sea
- Excretion of nutrients by rabbitfish increases the abundance of planktonic microbes
- Excretion of nutrients by rabbitfish changes the diversity of planktonic bacteria
- Excretion of nutrients by rabbitfish changes the nutrient content of macroalgae
- Egestion of feces by rabbitfish changes the diversity of benthic bacteria
- Egestion of feces by rabbitfish changes the diversity of benthic bacteria