1-3-2023

Selective and Differential Feeding on Marine Prokaryotes by Mucous Mesh Feeders

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Title: Selective and differential feeding on marine prokaryotes by mucous mesh feeders

Running title: Microbial prey

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Keywords
Microbial mortality, salp, pteropod, grazing

Conflicts of Interest
The authors declare no competing financial interests.

Originality-Significance Statement
Observations of marine microbial mortality exceed predictions of cell loss from known microbial predators including viruses, protists, and small crustaceans. Our work shows that mucous mesh grazers consume a variety of marine prokaryotes and select between closely related microbial lineages and similar cell sizes. This is the first study to show that *Prochlorococcus* may evade a major source of microbial mortality in the ocean and that planktonic archaea are consumed by macrozooplankton grazers. Discovery of these feeding relationships alters understanding of top-down processes that shape microbial community and function.

Summary

Microbial mortality impacts the structure of food webs, carbon flow, and the interactions that create dynamic patterns of abundance across gradients in space and time in diverse ecosystems. In the oceans, estimates of microbial mortality by viruses, protists, and small zooplankton do not account fully for observations of loss, suggesting the existence of underappreciated mortality sources. We examined how ubiquitous mucous mesh feeders (i.e. gelatinous zooplankton) could contribute to microbial mortality in the open ocean. We coupled capture of live animals by blue-water diving to sequence-based approaches to measure the enrichment and selectivity of feeding by two coexisting mucous grazer taxa (pteropods and salps) on numerically dominant marine prokaryotes. We show that mucous mesh grazers consume a variety of marine prokaryotes and select between coexisting lineages and similar cell sizes. We show that *Prochlorococcus* may evade filtration more than other cells and that planktonic archaea are consumed by macrozooplanktonic grazers. Discovery of these feeding relationships identifies a new source of mortality for Earth's dominant marine microbes and alters our understanding of how top-down processes shape microbial community and function.

feeding mesh. Thecosome pteropods produce an external mucous bubble and hover in place, relying on passive sedimentation of particles onto the mesh.

Bluewater SCUBA enabled capture of the grazers without damage for the assessment of gut prey contents and associated microbial communities quickly after collection. Using 16S rRNA gene surveys of microbial taxa in guts relative to the seawater prey field, we addressed whether mucous grazers feed selectively on distinct populations of microbial prey and how selected prey differ between the coexisting grazers. We also applied these techniques to salp fecal pellets to track the fate of consumed prey and role in the microbial loop. This approach can address the distinct impacts of mucous grazer feeding on microbial community structure, ecology, participation in carbon export, and the microbial loop. Future work will address mucus pore sizes, chemical composition, and feeding fluid mechanics that underlie these observations of differential and selective feeding by pelagic predators.

Experimental Procedures

Study site and SCUBA-based sampling

Seawater and animal samples were collected from the western edge of the Gulf Stream (26° 43' 93" N, 79° 59' 15" W) in September 2019, 5-8 km east of West Palm Beach, Florida. Water depth at the study site was 180-220 m and the temperature was 30° C. Bluewater SCUBA techniques were used to capture living salps or pteropods (Figure 1) in clear 1L polycarbonate jars. Accompanying seawater samples to assess the prey field were collected similarly in 1L polycarbonate jars in the area surrounding the collected animals, on the same dive (i.e. within 45 minutes). All samples were collected during daylight in the upper 15 m. Jars with animals and jars with seawater were brought on the deck of a 10 m dive vessel for processing. Within 30 minutes of divers surfacing, samples were archived or processed as follows. Salps and pteropods were gently poured onto a metal sieve then rinsed with 0.2 μm filtered seawater. Each gut was then removed with dissecting scissors, avoiding as much of the gelatinous tissue as possible. Guts were placed

into sterile bead-beating tubes with 0.55 mm and 0.25 mm sterile glass beads and stored on dry ice until archiving at -80 °C in a shore-based lab. Salp fecal pellets were sampled from different salp specimens incubated in jars for approximately 1-hour after collection. Fecal pellets were collected on a mesh sieve (500 µm), rinsed with 0.2 µm filtered seawater, then stored as above. Jars containing seawater collected near sampled animals were transported on blue ice to the shore-based lab. For flow cytometry samples, 2 mL of seawater was fixed at a final concentration of 0.125% TEM grade glutaraldehyde (Tousimis), incubated at room temperature for 10 minutes, then flash frozen in ethanol cooled with dry ice. Seawater DNA samples were taken by peristaltic pumping onto 0.2 µm membrane filters and were stored on dry ice until archiving at -80 °C.

DNA extractions and PCR with universal 16S rRNA gene primers.

DNA was extracted with the DNeasy Plant Tissue Mini Kit (Qiagen) with the following modifications. Salp guts, salp fecal pellets, and pteropod tissues were ground with a sterile disposable pestle (Axygen, Tewksbury, USA) for 3 minutes prior to extraction. All samples, including seawater, were lysed by bead beating with 0.55 mm and 0.25 mm sterile glass beads at 30 Hz for 2 minutes after addition of lysis buffer, freeze-fractured 3 times, incubated with a final concentration of 2 mg/mL Proteinase K (VWR Chemicals, Solon, OH, USA) for 1 hour at 55 ˚C, and then incubated with a final concentration of 0.9 mg/mL RNase A for 10 minutes at 65˚C. To minimize amplification of eukaryotic host DNA, the primer pair 515F-Y/806R was chosen to amplify the 16S rRNA V4 hypervariable region with conditions as published (Caporaso et al. 2018). Reactions were performed with 0.5-2 ng of DNA using the QuantaBio 5Prime HotMasterMix (Qiagen Beverly, MA, USA). To overcome PCR inhibition in salp samples, bovine serum albumin (BSA) was added to the salp PCRs, see details below. The Agilent Bioanalyzer High Sensitivity Kit (Agilent Technologies, Waldbronn, Germany) confirmed amplicon size. Triplicate PCRs from each sample were pooled, cleaned with magnetic beads, and paired-end

sequenced (2 x 300 bp) with Illumina MiSeq v.3 (Illumina, San Diego, USA). Sequences were deposited in the Sequence Read Archive (SRA).

Identification of amplicon sequence variants (ASVs)

16S rRNA sequence reads were processed using *dada2* (Callahan et al. 2016) and *phyloseq* (McMurdie and Holmes 2013). Sequences were quality controlled using *filterAndTrim()* with truncLen set to 190 (forward reads) and 160 (reverse reads), maxEE was set to 3, and maxN set to 0 to eliminate low quality base calls. Forward and reverse primers were trimmed from all reads. Error learning, sample inference, and merging of paired-end reads were done with *dada2* default settings to yield unique amplicon sequence variants (ASVs). Chimeric ASVs were removed with the "consensus" method. The reference database "RefSeq-RDP16S_v2_May2018" was used to assign taxonomy to the ASVs. *phyloseq* was used to connect ASV sequence counts per sample to taxonomic data and metadata. Sequence abundances were standardized to the median sequencing depth of all samples ("standardized relative abundance") (Supplemental Figure 1) without rarefying (McMurdie and Holmes 2014). ASV sequences are provided in Supplemental Materials. Sequence data are deposited in NCBI's Sequence Read Archive under the BioProject ID PRJNA867417.

Diversity metrics

Diversity analyses were done with *phyloseq* (McMurdie and Holmes 2013). For alpha diversity analysis, *plot_richness* calculated and plotted Shannon diversity levels for each sample type. Bray Curtis (BC) dissimilarities for beta diversity analysis were calculated with *ordinate* using method "NMDS" and distance "bray". Homogeneity of variances between samples was tested by performing ANOVA on the multivariate dispersions calculated with the function *betadisper* from the *vegan* package (*p-value* = 0.08, not significant) prior to analysis of similarity (ANOSIM) (Oksanen, E. Jari et al. 2019). The ANOSIM test was used to determine the significance and *p-values* for sample grouping by sample type (salp, seawater, etc.).

Prey analysis

To study the feeding of salps and pteropods on microbial prey, we considered a limited subset of the ASVs as potential prey taxa. The potential prey ASVs we chose are well-characterized, abundant, and known free-living marine microorganisms that we identified from the ASV taxonomic assignments. In addition, the prey ASVs had to be abundant in our seawater samples. The identification of prey ASVs was also confirmed by BLASTn (Johnson et al. 2008). The potential prey we chose included the heterotroph *Pelagibacter (SAR11)*, marine picocyanobacteria (*Prochlorococcus* and *Synechococcus*), marine planktonic archaea (Euryarchaeota and Thaumarchaeota), and multiple lineages of eukaryotic phytoplankton (identified by their chloroplast sequences amplified by the 16S rRNA gene universal primers including diatoms, prymnesiophytes, and chlorophytes). ASVs belonging to each prey group were summed in order to focus on taxonomic groups of prey, rather than individual ASVs.

We further considered prey retention in two ways. First, we interpreted the presence of all prey species in our gut samples as evidence of retention through feeding. The possibility that the prey ASVs were present due to contamination from seawater was mitigated by the dissection of gut tissue away from the tissue of the animals that had direct contact with seawater. Second, because the dataset is compositional, we could not compare the relative abundance of prey ASV sequences between the seawater and gut samples to indicate selectivity (McMurdie and Holmes 2014; Gloor et al. 2017; Kim et al. 2017; Carr et al. 2019). Instead, we compared the relative abundance of prey taxa to each other within the guts vs. within the seawater. To do this, we normalized the prey sequence counts in the individual gut samples by dividing by the mean counts from all seawater samples (n=5). We then compared the normalized means of each prey taxa to other prey taxa in the guts to determine if the relative proportions of the prey to each other were different in the guts than they were in the seawater. We only considered prey that were present in all the salps or all the pteropods. One salp sample was determined to be an outlier as it was dominated by *Streptophyta*

grazers on primary production, phytoplankton communities, and carbon export and cycling (Luo et al. 2020, 2022). Currently, there is only limited evidence that this grazing impact extends to the smallest, but more numerous, members of microbial communities, including marine Bacteria and Archaea.

One reason for limited understanding of mucous mesh grazer predation on marine prokaryotes is that traditional microscopy techniques miss prokaryotic prey in feeding organs due to small cell sizes and non-distinct morphologies. In addition, it is difficult to sample grazers in the remote tropical and subtropical open ocean where prokaryotes dominate the microbial community. Mucous mesh grazers are patchy in distribution, bloom timing is difficult to predict, and the grazers' extreme fragility requires open-water SCUBA-based techniques for non-destructive sampling (Hamner et al. 1975; Alldredge and Madin 1982; Deibel and Lowen 2012). Despite these challenges, a handful of studies have documented capture of bacteria, archaea, and even viruses by mucous grazers (Sutherland and Thompson 2022). Analysis of pyrosome feeding organs demonstrates retention of picocyanobacteria (Thompson et al. 2021). For salps, direct *in situ* sampling and incubations demonstrate retention of small heterotrophic bacteria (e.g. *Pelagibacter*) (Dadon-Pilosof et al. 2019). Together with discovery of very small mesh pore sizes of salps (Bone et al. 2003; Sutherland et al. 2010), these studies suggest retention of bacterial and archaeal prey, and inspire studies to further address whether mucous grazer predation can account for unexplained microbial loss from major surface ecosystems.

To address the impact of mucous mesh grazers on marine microbial communities, we examined microbial prey of two coexisting mucous mesh grazers: *Iasis cylindrica* – a salp, and *Corolla spectabilis* – a thecosome pteropod (Figure 1). We worked in the Gulf Stream, which contains microbial communities that represent the oligotrophic open-ocean gyres of the tropical and subtropical Atlantic Ocean (Wang et al. 2019). Salps and pteropods both use mucous feeding meshes to concentrate food particles, but they employ different filtration strategies (Conley et al. 2018). Salps use muscular pumping to move fluid into the barrel-shaped body and through the

and was statistically different from the other 6 salp samples in analysis of beta diversity, so this analysis was performed with 6 salp samples to address the prey question. Future work using quantitative assays (i.e. quantitative PCR) on multiple prey types in grazer guts and surrounding seawater could avoid some of the issues with compositional datasets (Frischer et al. 2014, 2021).

Prey sizes were also examined in the context of their retention in guts. Minimum dimensions were based on previous publications including: *Prochlorococcus* 0.55 μm (Casey et al. 2019), *Synechococcus* 1.1 μm (Waterbury et al. 1979), Euryarchaeota 0.7 μm (Orellana et al. 2019), *Pelagibacter* 0.15 μm (Morris et al. 2002), *T. pseudonana* 7.5 μm (Ribalet et al. 2019), and *I. rotunda* 3 μm (Reynolds 1974).

Flow cytometry

Seawater (i.e. prey field) prokaryotic and eukaryotic phytoplankton abundances were measured via flow cytometry using a BD Influx high-speed cell sorter (BD Biosciences) equipped with a 488 nm excitation laser and small particle detector. Data collection was triggered on forward scattered light (FSC). Red fluorescence (692/40 bandpass filter) and orange fluorescence (572/27 bandpass filter), side scatter (SSC), and FSC were collected for each particle. Three phytoplankton populations were enumerated including *Prochlorococcus*, *Synechococcus*, and pigmented picoeukaryotes (PPE) all relative to 1 μm yellow-green polystyrene beads (Polysciences) with gating as shown in Supplemental Figure 2. Gating of populations was done in FlowJo version 7.6.5 (TreeStar). Instrument flow rate was obtained by measuring the volume removed from triplicate sheath-filled sample tubes over time. The analyzed volume of each seawater sample was calculated by multiplying the time the sample was analyzed by the flow rate. Seawater cell concentrations were calculated by dividing cell counts by analyzed volume for each sample.

Results

Amplicon sequence variants recovered from salps, pteropods, and seawater

In total, 1,884,205 quality-filtered sequences were recovered from salp guts, salp fecal pellets, pteropod guts, and surrounding seawater samples. The median number of sequences per sample was 88,460 with the lowest number of sequences recovered from a pteropod sample (12,427) and highest number of sequences recovered from a salp fecal pellet (274,672) (Supplementary Figure 1). One pteropod DNA sample did not produce any quality-filtered reads and was eliminated from the analysis. From these sequences, we discovered 2,566 unique amplicon sequence variants (ASVs) across 20 phyla (Supplemental Figure 4). Most ASVs belonged to Proteobacteria (n=1190), Cyanobacteria or Chloroplasts (n=271), or Bacteroidetes (n=208) (Supplemental Figure 4).

Diversity of salp and pteropod microbial communities relative to surrounding seawater

We examined alpha and beta diversity metrics to determine how the microbial communities of the mucous mesh grazers differed from the seawater that surrounds them. Diversity analyses showed significant differences between the salps, pteropods, and surrounding seawater (Figure 2). Shannon diversity (diversity and evenness, alpha) was distinct by sample type (Kruskal-Wallis global *p-value* < 0.05, Figure 2A). Compared to seawater, Shannon diversity was lower for the salp (*p-value* = 0.048) and pteropod guts (not significant, due to a low number of animals sampled). Salp and pteropod gut Shannon diversity were not significantly different from each other (*p-value* > 0.05). For salp fecal pellets, Shannon diversity was substantially lower than for salp guts (*p-value* <0.05, Figure 2A). Analysis of beta diversity using Bray Curtis dissimilarities visualized by non-metric dimensional scaling (NMDS) showed distinct microbial community structures for pteropod and salp guts (Figure 2B). The fecal pellet microbial community structure was also distinct from all other sample types, including the salp guts (Figure 2B). Overall, the microbial communities were strongly partitioned by the sample source (ANOSIM R statistic 0.9771 and significance 0.001).

Dominant microbial taxa of salps and pteropods

We examined the microbial taxa behind the distinct diversity patterns by looking at the relative abundance of the top ten most abundant ASVs across each type of sample (Figure 3). Seawater sequences were dominated by marine picocyanobacteria (*Prochlorococcus* and *Synechococcus*) as well as the ubiquitous heterotrophic marine bacterium *Pelagibacter ubique* (SAR11). The numerical dominance of the picocyanobacteria was supported by flow cytometry analysis (Supplemental Figure 3). Representatives of unclassified Alpha- and Gamma-proteobacteria were also relatively abundant, as were sequences matching the Family *Flavobacteriia*. The major seawater taxa are similar to previous observations of microbial community composition in the Gulf Stream and subtropical open-ocean gyres (Wang et al. 2019).

Salp guts were dominated by several *Synechococcus* ASVs (approximately 30% of sequences) and three ASVs from the Planctomycetia (approximately 50% of sequences), the largest class of the phylum Planctomycetes (Vitorino and Lage 2022). The three Planctomycetia ASVs were similar to the *Blastopirellula*, *Thermogutta*, and *Planctomycetaceae* genera. Also relatively abundant in salp guts, but not dominant, were chloroplast sequences from the centric diatom *Thalassiosira pseudonana* and sequences matching the Alphaproteobacterium genus *Rhodobacteraceae*. The dominance of Planctomycetes in association with salps is consistent with the ubiquity of the lineage, adaptations to diverse habitats, and is consistent with the microbiome composition of other pelagic tunicates (Wiegand et al. 2018; Thompson et al. 2021).

Salp fecal pellets were dominated by two distinct *Vibrio* ASVs. ASV1 matched *Vibrio rotiferianus* and ASV26 matched *Vibrio fortis*. These *Vibrio* ASVs were poorly represented in salp guts and seawater. Other sequences in the salp fecal pellets included *Synechococcus*, *Alteromonas*, and *Pelagibacter ubique*.

Pteropod microbial community structure was distinct from salps and seawater, though the number of animals sampled was very small (n=2) due to patchy distribution and limited dive time (Figure 3). However, while distinct in terms of the community structure and balance of dominant

ASVs, pteropod gut ASVs were taxonomically similar to ASVs recovered from the salp guts. Planctomycetia ASVs were prominent in the pteropod guts, with representatives from *Blastospirellula, Planctomycetaceae, and Thermogutta*, as in the salp guts. In one of the pteropods, the *Blastopirellula* ASV comprised more than 80% of the sequences. *Synechococcus* and *Pelagibacter* ASVs were also present in the pteropods, but less relatively abundant than they were in the salp guts (Figure 3).

Diverse prokaryotic microbial prey in salp and pteropod guts

To examine feeding of salps and pteropods on microbial prey, especially Bacteria and Archaea, we defined a set of known free-living microbial lineages from the recovered ASVs as potential prey taxa. Prey ASVs included *Pelagibacter*, marine picocyanobacteria (*Prochlorococcus* and *Synechococcus*), marine planktonic archaea (Euryarchaeota and Thaumarchaeota), and a suite of eukaryotic phytoplankton (recovered through their chloroplast sequences amplified by the 16S rRNA gene universal primers) (See Experimental Procedures). Cytometric (Supplemental Figure 3) and sequence-based methods (Figure 4) both showed the seawater prey field as dominated by *Prochlorococcus* and small non-pigmented cells, with *Synechococcus* and pigmented eukaryotes present at lower absolute (or relative) abundances.

Salp and pteropod guts contained a variety of prey species. Prey belonged to all three domains of life, including planktonic Archaea (Euryarchaeota and a little Thaumarchaeota), which are thought to evade grazing due to their smaller size (~0.7 μm) (Orellana et al. 2019). Retained prey taxa ranged in size from small heterotrophs, (i.e. *Pelagibacter*) to larger phototrophic eukaryotes (e.g. Prymnesiophytes, Cryptophytes, and Bacillariophytes). In all gut samples, retained prey were dominated by the picocyanobacterium *Synechococcus*. However, *Synechococcus* was not the dominant member of the seawater microbial community (Supplemental Figure 3). This is one of very few studies of prokaryotic prey in pteropods (Thibodeau et al. 2022) as previous studies targeted eukaryotic taxa (Gilmer and Harbison 1986).

Salp and pteropod selective feeding

Next, we compared the relative proportions of prey taxa to each other in the guts and in the seawater to address prey selection (Figure 5). We performed this analysis for seven prey taxa that were present in seawater samples and in the majority of salp samples. Due to the constraints of working with compositional data (McMurdie and Holmes 2014; Gloor et al. 2017), we did not interpret positive and negative y-axis values as enriched or depleted in the guts, respectively (See Methods). Instead, we compared the relative proportions of prey taxa to each other in the gut samples and in the seawater samples.

Overall Kruskal-Wallis tests across taxa showed significant differences across the prey taxa in their proportions to each other in the salp guts relative to their proportions in the seawater (*p-value << 0.01*) (Figure 5B). Due to low numbers of animals sampled, differences across microbial taxa were not significant for pteropods (Figure 5A).

The two picocyanobacteria, *Prochlorococcus* and *Synechococcus*, were present at significantly different proportions in the salp guts compared to their proportions in the seawater samples (*p-value* <<0.05). The ratio of *Synechococcus* to *Prochlorococcus* was higher in the guts than in the seawater, despite *Prochlorococcus* being more abundant in the seawater prey field (Supplemental Figure 3). Similarly, the ratio of *Synechococcus* to *Pelagibacter* was higher in the guts than in the seawater. The ratio of *Pelagibacter* to *Prochlorococcus* was also higher in the guts than in the seawater (*p-value* <<0.05). Euryarchaeota were more relatively abundant than *Pelagibacter* and *Prochlorococcus* in the guts than they were in the seawater, but Euryarchaeota were less relatively abundant in the guts than the similarly-sized *Synechococcus*. Two picoeukaryotic phytoplankton, the diatom *Thalassiosira pseudonana* and the prymnesiophyte *Imantonia rotunda,* were present at higher proportions relative to *Prochlorococcus* and *Pelagibacter* in the salp guts than in seawater (*p-value* <<0.05), but at lower proportions to

Synechococcus. Prey size from the literature (minimum dimension) did not correspond to retention (Figure 5B, top).

The low number of pteropods sampled (n=2), prohibited robust statistical analysis and comparison between taxa. Indeed, the Kruskal-Wallis overall test did not support differences between prey taxa (p-*value* >> 0.05). However, sequences from *Synechococcus*, *Pelagibacter*, and *T. pseudonana* were present in both pteropod guts (Figure 4, Figure 5A).

Discussion

Salps and pteropods graze on marine prokaryotes

Viral lysis and protistan grazing do not fully account for observed losses of marine prokaryotes from the tropical and subtropical surface oceans, suggesting unrecognized sources of mortality (Talmy et al. 2019; Beckett et al. 2021). These missing mortality factors limit the ability of ecosystem models to link bottom-up and top-down processes in predictive frameworks that test how marine microbial communities, and their carbon cycle contributions, will change with shifts in oceanic conditions. This study shows that mucous mesh grazers are a source of mortality for a variety of ubiquitous and numerically-abundant bacteria and archaea in the surface waters of the Gulf Stream. Marine picocyanobacteria *Prochlorococcus* and *Synechococcus* were a component of the prokaryotic prey in salp guts from the Gulf Stream, which are waters representative of major subtropical ocean gyres where these taxa make substantial contributions to primary production (Berthelot et al. 2021). The ubiquitous marine heterotroph *Pelagibacter* was also in gut samples of both pteropods and salps. Though *Pelagibacter* may escape predation with its unique surface properties (Dadon-Pilosof et al. 2017), our study suggests that some cells are still retained and removed from the surface ocean. *Prochlorococcus* was retained even less than *Pelagibacter*, which is the first evidence that *Prochlorococcus* may partially escape a major mortality source in the ocean. Planktonic archaea were another component of the salp diet. Though understanding of their ecology and physiology is incomplete, Euryarchaeota appear to be small free-living cells (~0.7 μm)

(Orellana et al. 2019) that compose a major part of microbial biomass in the ocean (DeLong 1992), and contribute to distinct biogeochemical processes. Their removal by large mucous mesh grazers alters the picture of how planktonic archaea link to marine food webs. This work, combined with results from experiments on cultivated grazers, artificial prey, measurements of mesh pore sizes, and studies from coastal environments, suggests a role for mucous grazers in marine microbial mortality and evolution (Sutherland et al. 2010; Dadon-Pilosof et al. 2019; Stukel et al. 2021; Fender et al. 2022). As mucous mesh grazers are ubiquitous, and bloom rapidly in response to change, this trophic interaction likely has a global role in shaping nutrient cycling, microbial community ecology, evolution, and cross-scale ecosystem structure.

Salp feeding is selective

Comparing the ratios of prey taxa to each other in the guts and seawater revealed changes in the relative abundances of prey, suggesting selective feeding (Figure 5A-B). Though size is an important property governing particle capture by salps (e.g. Fender et al, 2022), mounting evidence indicates that not only can mucous mesh grazers capture particles smaller than mesh openings at appreciable rates (Conley et al. 2018) but particles of similar size are not collected at the same rates (Dadon-Pilosof et al. 2019). In the present study, *Prochlorococcus* (~0.55 μm; (Casey et al. 2019)) was proportionally much less abundant in the guts than other prey types of similar size (Figure 5B, top). *Synechococcus* (L:W ~1.1:2 μm; (Waterbury et al. 1979)) was the second most abundant prey type proportionally, followed by Euryarchaeota (~0.7 μm; (Orellana et al. 2019)). *Pelagibacter* (L:W ~0.15:0.65 μm; (Morris et al. 2002)) was also concentrated at a much lower proportion than *Synechococcus*. Collectively, these data indicate that selective feeding is occurring among particles in the micron and submicron range, many of which are likely smaller than the mesh openings (~ 1 μm: Bone et al. 2003; Sutherland et al. 2010) (Figure 5B). Size-independent feeding is consistent with recent salp-feeding studies showing that salp species select for particular microbes. Antarctic salps preferred dinoflagellates over other microbes and there were differences in the dinoflagellate

species selected (Metfies et al. 2014), and salp species from oligotrophic Mediterranean waters preferred picoeukaryotes in the 1 μm range over both smaller cyanobacteria and larger nanoeukaryotes (Dadon-Pilosof et al. 2019).

Salp gut data further suggest that feeding is not taxonomy based. *Prochlorococcus* to *Synechococcus* relative abundance was lower in salp guts than in seawater, suggesting more retention of *Synechococcus* by the salps, despite *Prochlorococcus* being more abundant in the background seawater (Supplemental Figure 3). This result suggests that selection is occurring even between these two related cyanobacteria and that *Prochlorococcus* may be escaping this grazing pressure. In total, evidence suggests selective feeding, but there is yet to be consensus on the microbial characteristics that govern retention by mucous mesh grazers.

We considered caveats of our approach to determine selectivity of feeding. One caveat associated with exploring selectivity is the possibility that different prey types are digested at different rates within the gut. Also, robustly quantitative approaches, such as qPCR aimed at specific prey groups, will improve understanding of selectivity not possible with compositional approaches such as sequencing. Differential digestion rates could deplete the DNA of specific consumed prey in the sequence databases from gut samples, shifting their abundances in the guts whether measured through cell-based or molecular means. Differential proportions of prey in the gut could also happen through microbial means, as we observed fecal pellet colonization by rapidly growing species (i.e. *Vibrio*), that could deplete prey types relative to each other. Molecular techniques, such as those applied to doliolids and small crustaceans, offer a promising approach to quantify differential prey digestion in future studies (Durbin et al. 2012; Frischer et al. 2014). Another caveat we faced when comparing seawater at the sample site to the guts of salps and pteropods is the possibility that the animals fed outside our sample area. Thus, their gut content would not reflect the prey field we sampled. This is especially possible for salps, which are known to vertically migrate. Timed *in situ* incubations with controlled prey fields could strengthen the study of selectivity.

Salps as a common enemy to marine prokaryotes

Defining interactions between microorganisms in the marine environment is a major challenge, but critical to advance understanding of the assembly and function of microbial community structure, carbon flow, and biogeochemical cycling (Azam and Malfatti 2007; Lima-Mendez et al. 2015). Apparent competition (also called shared predation) is an indirect interaction that results from two prey species sharing a common predator (Holt and Bonsall 2017). This indirect interaction impacts species distributions, abundances, and dynamics. For example, in a scenario with two prey types (A and B), when prey species A becomes more abundant, its predator thrives, which leads to enhanced predation and a negative effect on prey species B.

Recent work shows that incorporating apparent competition between *Prochlorococcus* and heterotrophs (or *Synechococcus*) into ecosystem models explains the absence of *Prochlorococcus* at high latitudes and its attrition over depth (Follett et al. 2022). Shared predation could be imposed by a range of different grazers. For example, some broad-range cyanophage may infect multiple lineages of marine picocyanobacteria (Sullivan et al. 2003). Protists can also be shared predators, as shown by tracking stable isotope labeled *Prochlorococcus* and *Synechococcus* into common lineages of mixotrophic grazers (Frias-Lopez et al. 2009). Our work demonstrates that mucous mesh grazers should also be examined as a source of shared predation between numerically-abundant marine microbes. Our data suggest retention of multiple microbial taxa by a common grazer. How these rates compare to removal through protistan grazing and viral lysis (Carlson et al. 2022) are unknown for the subtropical ocean. However, our data also suggest differential pressure on different microbial taxa by mucous grazers through selective feeding (Figure 5), with *Synechococcus* retained in salp guts more than *Prochlorococcus* and *Pelagibacter*. Likely patterns of selective removal depend on relative and absolute prey abundances, the mucous grazer taxonomy, life stage, and size (Frischer et al. 2021; Stukel et al. 2021). Continued work to couple clearance rates to feeding selectivity is needed for a greater range of mucous grazer taxa, ecosystem

conditions, and microbial community compositions. Such data will help resolve whether mucous grazers are generalist or specialist predators and will guide a framework not only for their impact on carbon cycle contributions (Stukel et al. 2021) but on the complex interactions between marine microbes (Lima-Mendez et al. 2015).

Filtration mechanisms

Though both filter feeders use a common strategy of concentrating prey on a mucous filter with a large surface area, this work shows that the details of the feeding mechanisms in salps and pteropods may result in different prey retention. Salps secrete an internal mucous mesh with a rectangular grid; mesh fibers are nanometers in diameter and mesh openings are ~ 1 μm (Bone et al. 2003). Muscular pumping in salps results in flow past the mesh at speeds of ~1 cm s^{-1} and submicron-sized particles are captured on the mesh fibers via direct interception (Sutherland et al. 2010). Unlike the organized rectangular mesh of salps, pseudothecosome pteropods have a diffuse mesh of 1 to 6 μm fibers with irregular spacing and fiber orientation that produce opening sizes range from 250 to 4000 μm (Gilmer 1974). The morphology and mechanics of filtration suggest that the volume filtered should be low relative to salps and that prey particles should be captured via sedimentation indiscriminately as long as the particles are larger than the mucous mesh openings. Our results show that this feeding behavior could impact the smallest and most numerous microbial cells in seawater (Figure 4-5A), suggesting that functional pore-sizes of pteropods may be smaller than previously thought. The possibility that pteropods sediment even small microbial taxa complements recent observations that marine picocyanobacteria sink, despite their small size and low Reynolds number fluid interactions (Aguilo-Ferretjans et al. 2021). It is also possible that these small microbial taxa associate with larger food particles (i.e. detritus and other particles), which is consistent with picocyanobacteria and *Pelagibacter* detection on larger suspended particles (Karsenti et al. 2011; Boeuf et al. 2019). Indeed, microbial diversity of pteropod guts was more similar to the seawater than were salp guts (Figure 2A). Whether through association with

large particles, or ingested as free-living cells, our results suggest that carbon from small prokaryotic taxa fuels pteropods in subtropical oceans.

The selective feeding on marine microbial communities by salps and pteropods support the hypothesis that mesh filtration is not due to simple sieving, where fluid and associated food particles travel in a straight path through the filter. The selection between microbial prey types suggests that, instead, filtration involves complex flow patterns past the filter mesh and small-scale interactions at the level of the filter mesh. Fluid does not pass directly through the filter of salps; it moves in a circular pattern past the mesh (Sutherland et al. 2010) and is likely governed by tangential flow filtration, similar to benthic ascidians (Conley et al. 2018). Pteropod fluid handing is much less well understood. Next steps will require further imaging of filter meshes—especially *in vivo* (e.g. Conley et al. 2017)—together with fluid visualizations and experiments with particles of known sizes, shapes and surface properties that represent dominant marine prokaryotes.

Ecosystem impacts – the fate of the particles

After particles are grazed by mucous mesh grazers, they have different fates depending on grazer taxon and therefore, divergent contributions to biogeochemical cycling. The microbial community of salp fecal pellets provides an insight into the link between consumed prey and the marine carbon cycle. Salp fecal pellets sink quickly, contributing to vertical export of carbon and the biological pump (Bruland and Silver 1981; Komar et al. 1981, Caron et al. 1989; Cherry et al. 1978). However, some of this carbon may be recycled in the surface waters through bacterial colonization (Gowing & Silver 1983, Jacobsen & Azam 1984, Caron et al. 1989). The short generation times and versatile metabolism of *Vibrio spp*. (Zhang et al. 2018), suggests that remineralization of the fecal pellet material begins immediately in the surface waters. This observation is consistent with measurements of high turnover (and low export) of carbon from salp fecal pellets in a high latitude system mixed layer (Pauli et al. 2021a). In contrast, including salps in global carbon models push surface ecosystems away from recycling and towards carbon export

(Luo et al. 2022). Future work will examine how selection between marine microbes, and fecal pellet composition, impact recycling and export of carbon from salp fecal pellets.

Mucous-feeding pteropods represent a different biogeochemical pathway. Mucous bubbles are typically consumed along with collected prey but when feeding pteropods are disturbed, mucous bubbles are discarded. These discarded aggregates, like salp fecal pellets, may become microbial islands that are colonized as they sink through the water column. Pteropod fecal pellets also exhibit high sinking rates relative to crustaceans: previous measurements from *C. spectabilis* were up to 1,800 m d^{-1} (Silver and Bruland 1981).

Conclusions

A picture is emerging of mucous grazers as key components of the microbial loop through removal of microbial prey. The mortality sources of microbes are dynamic in space and time and we still lack a basic understanding of the relative importance of different top-down impacts from various grazers. However, this work suggests that mucous mesh grazers, which are both widespread and abundant, graze even the smallest marine microbes, including Archaea, and may be key players in food webs. Future work could uncover the relative grazing impacts of salps, crustaceans, protists, and viruses in the ocean's vast subtropical regions, as done recently for salps in a high latitude setting (Stukel et al. 2021). Furthermore, published abundances of salps, pteropods and other gelatinous grazers are likely severe underestimates. Their fragile, watery bodies are frequently damaged via net-sampling and only a few imaging systems have fields of view that are large enough to encompass gelatinous macroplankton (Madin et al. 2006; Cowen and Guigand 2008; Katija et al. 2017). Parallel advances in quantifying mucous grazer distribution and abundance, and quantifying their rates of predation on a range of microbial prey, will illuminate the global role of these predators on microbial cells in the oceans.

Author contributions

Description of author contributions follows the "CRediT" taxonomy (Brand et al. 2015). Conceptualization: A.W.T and K.R.S.; Methodology: A.W.T, C.P.S.-T., K.R.S.; Formal Analysis: A.W.T, C.P.S.-T.; Investigation: A.W.T, C.P.S.-T., K.R.S.; Writing: A.W.T, C.P.S.-T., K.R.S.; Visualization: A.W.T.; Supervision: A.W.T.; Funding Acquisition: A.W.T and K.R.S.

Acknowledgements

We thank Marco Corrales, Jack Costello, Brad Gemmell, and Sean Colin for assistance with animal collection and SCUBA operations. We are grateful to the crew of the dive boat Ms. Jackie out of West Palm Beach, Florida, for safe and productive diving operations. We appreciate John Gale's work in processing the flow cytometry samples. We are grateful to Melissa Steinman for support in amplification of salp DNA by PCR. Thank you to two anonymous reviewers for constructive review the manuscript. Funding was provided by NSF-OCE 1851412 to AWT and NSF-OCE 1851537 and 2125408 to KRS.

Supplemental Materials

Supplemental File 1. Sequences of ASVs discovered (.fasta).

Figure and Table Legends

Figure 1. Mucous mesh grazers imaged *in situ* at the study site. A) Salp *Iasis cylindrica* with background subtracted. Image credit: Brad Gemmell. B) Pteropod *Corolla spectabilis*. Image credit: Linda Ianiello. Scale bars (approximate) are 1 cm.

Figure 2. Alpha (A) and Beta diversity (B) metrics for seawater, salp (*Iasis cylindrica*) and pteropod (*Corolla spectabilis*) guts, and salp (*I. cylindrica*) fecal pellets. For boxplots, the middle line is the median, the top line is the 75th percentile, and the bottom line is the 25th percentile. Whiskers extend to the largest and the smallest value within the interquartile range, respectively. Dots are the individual data points for each sample. The overall test of differences by sample type was performed with Kruskal-Wallis with pairwise comparisons performed by the Mann Whitney test with adjustments for multiple tests by the Holm method.

Figure 3. Distribution across samples, standardized relative abundance, and taxonomic identity of the top 10 ASVs from each sample type across all samples. The lowest known taxonomy down to Genus and Class-level (in parentheses) are represented by different colors of the ASVs, except for

Introduction

Marine microorganisms play an essential role in fueling marine ecosystems and driving nutrient and carbon cycling on global scales (Azam 1998; Strom 2008). Evidence is gathering for the importance of top-down processes in shaping and controlling these microbial communities in the global ocean (Morris et al. 2011; Sher et al. 2011; Worden et al. 2015; Seymour et al. 2017; Becker et al. 2019; Connell et al. 2020; Carlson et al. 2022; Li et al. 2022).

Microbial mortality is one top-down process that is not well understood. For example, ecosystem models suggest that known sources of microbial mortality in the ocean do not account for all observed cell loss, or death, from the surface ocean. In modeling carbon flow and loss in a coastal system, up to 25% of carbon losses are not accounted for by viral lysis, protistan grazing, and small zooplankton feeding (Talmy et al. 2019). Similarly, comparison of measured daily mortality rates to a simple ecosystem model of viral and protistan grazing pressure reveal unexplained mortality (Beckett et al. 2021). Research examining other sources of predation is one way to address this gap in our understanding and quantification of microbial mortality in the ocean.

Mucous mesh grazers (pelagic tunicates and thecosome pteropods) are one such set of predators that are understudied despite their high potential impact on marine microbial communities and ocean carbon cycling. These macrozooplankton use adhesive mucous nets to capture food particles orders of magnitude smaller than themselves. Strong evidence exists for feeding of mucous mesh grazers on eukaryotic microbes, especially phytoplankton, through 18S rRNA gene sequencing of gut tissues (Frischer et al. 2021; Pauli et al. 2021b; Thibodeau et al. 2022), microscopy (Silver and Bruland 1981; Stukel et al. 2021; Thompson et al. 2021; Fender et al. 2022), flow cytometry (Dadon-Pilosof et al. 2019; Stukel et al. 2021; Thompson et al. 2021), and laboratory- or culture-based experiments with model eukaryotic prey (Selander and Tiselius 2003; Troedsson et al. 2007). The ubiquity of these filter feeders in the oceans (Bednaršek et al. 2012; Lucas et al. 2014) and their ability to restructure pelagic foodwebs when they bloom (Alldredge and Madin 1982; Brodeur et al. 2018), suggest important global impacts by mucous

recovered Eukaryotic phytoplankton ("Chloroplast"). Abbreviations: seawater (SW), fecal pellet (FP), pteropod (Pt).

Figure 4. Standardized relative abundance of potential prey taxa relative abundance across all samples. Potential prey taxa include abundant and free-living microbial lineages (See Experimental Procedures). ASVs present in only 1 sample are not presented. Abbreviations: *P. ubique* (*Pelagibacter ubique*), *T. pseudo*. (*Thalassiosira pseudonana*), *I. rotunda* (*Imantonia rotunda*), pteropod (Ptero.).

Figure 5. Comparison of proportions of prey taxa to each other in pteropod (A) and salp (B) guts normalized to prey proportions in the seawater prey field. Each dot is an individual gut sample normalized to seawater means of that prey taxa. The overall test of differences by sample type was performed with Kruskal-Wallis with pairwise comparisons performed by the Mann Whitney test with adjustments for multiple tests by the Holm method (**, p-value < 0.01; *, p-value < 0.05). Note that y-axes of A and B are on different scales. B, inset: Size (i.e. minimum dimension) of representative cultures of prey types. See Experimental Procedures for references. Abbreviations: *Synechococcus* (*Syn.*), *Prochlorococcus* (*Pro.*), *Pelagibacter ubique* (*P.ubique*), *Thalassiosira pseudonana* (*T. pseudo*), *Imantonia rotunda* (*I. rotunda*), Euryarchaeota (Eury.). For boxplots, the middle line is the median, the top line is the 75th percentile, and the bottom line is the 25th percentile. Whiskers extend to the largest and smallest values up to 1.5 times the inter-quartile range. Outliers, points beyond 1.5 times the inter-quartile range, are represented by points.

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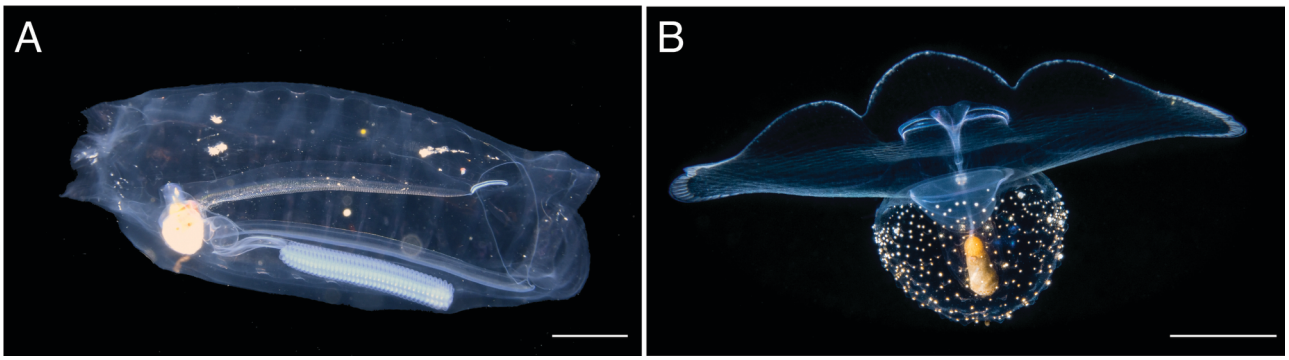

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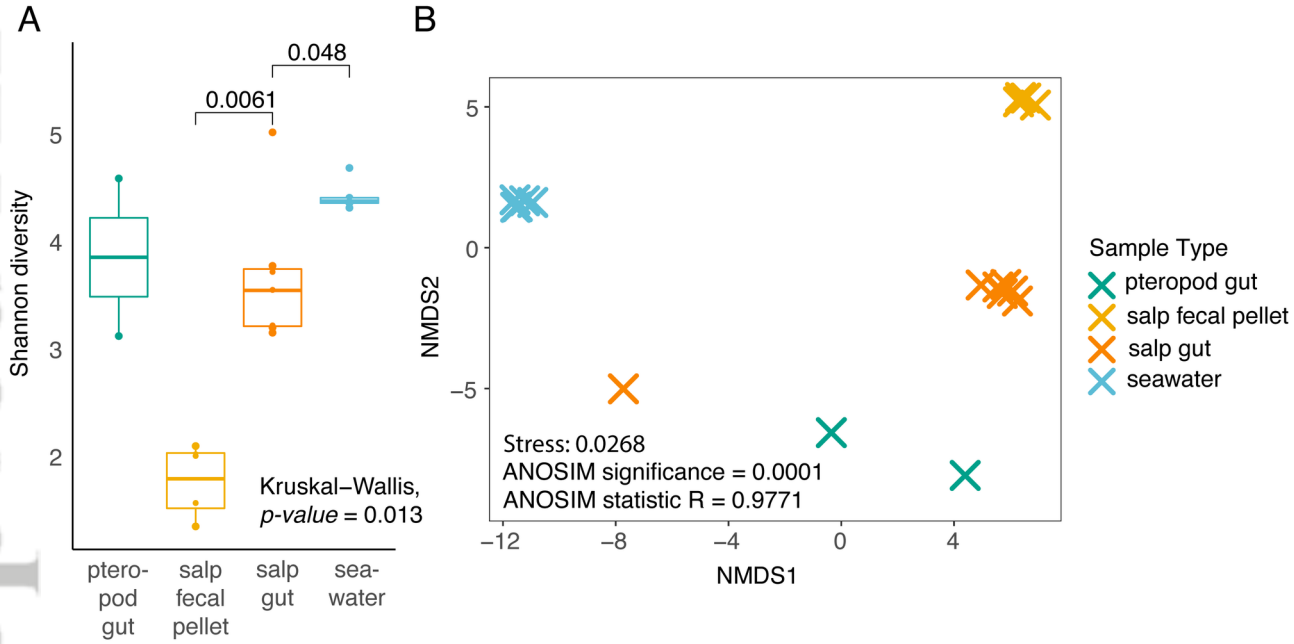

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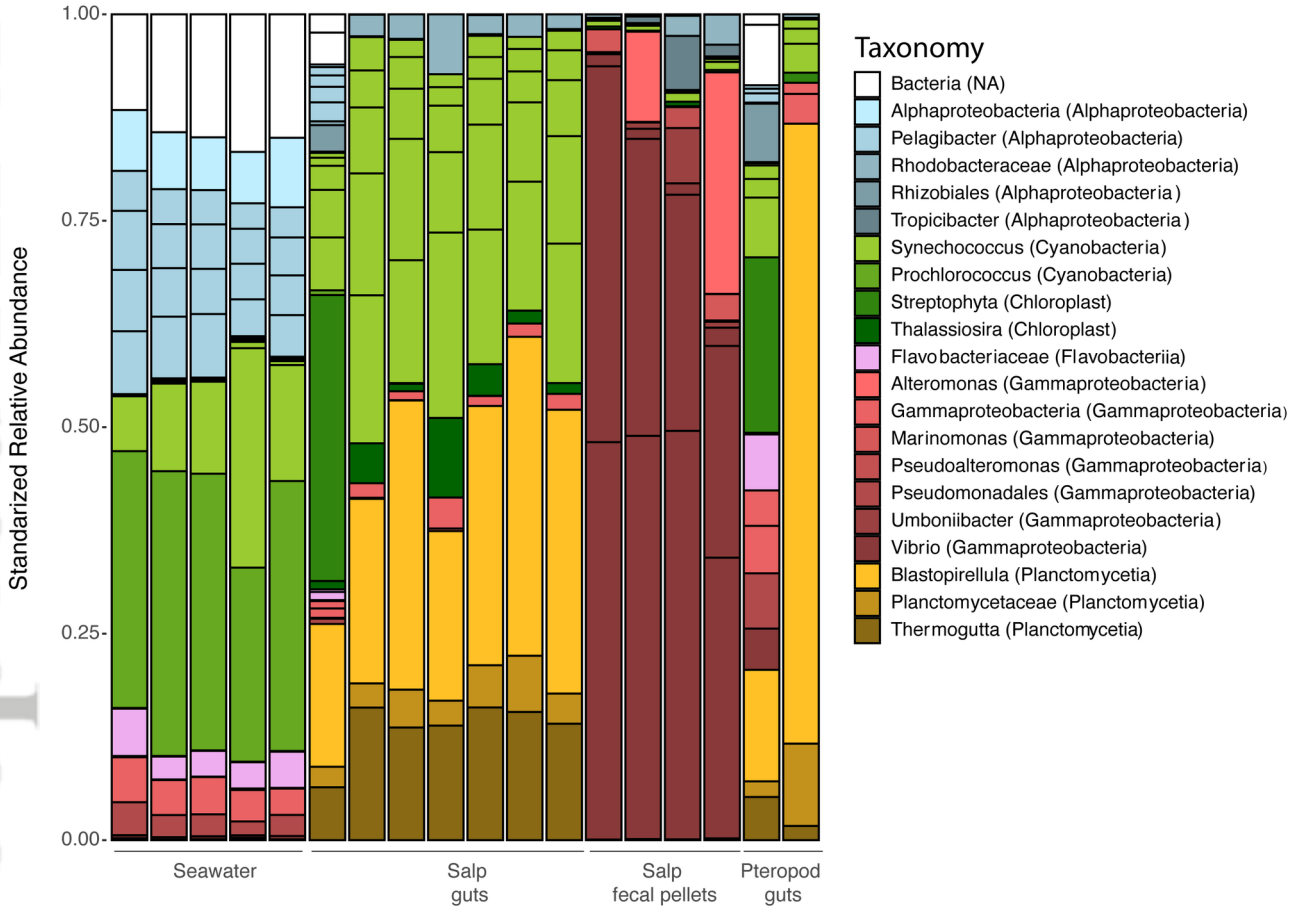

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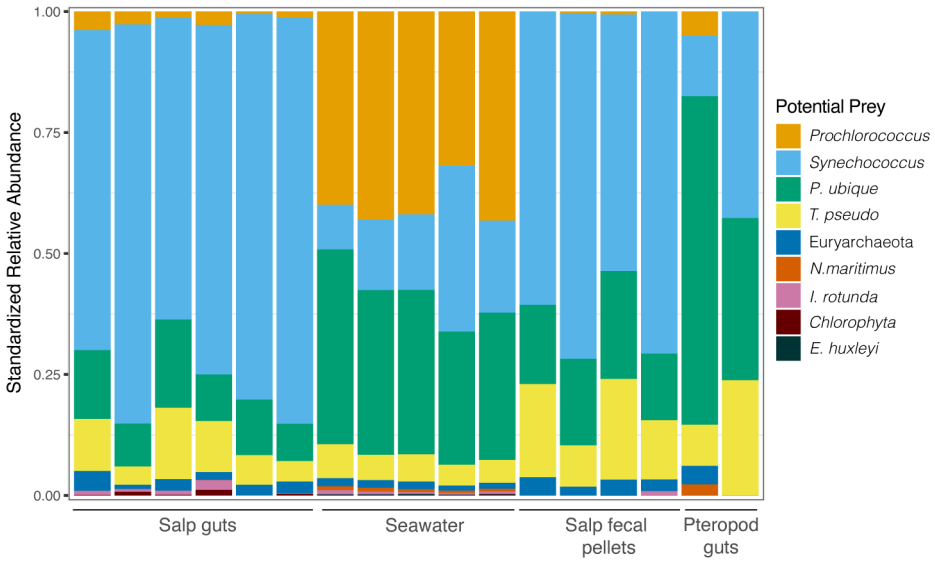

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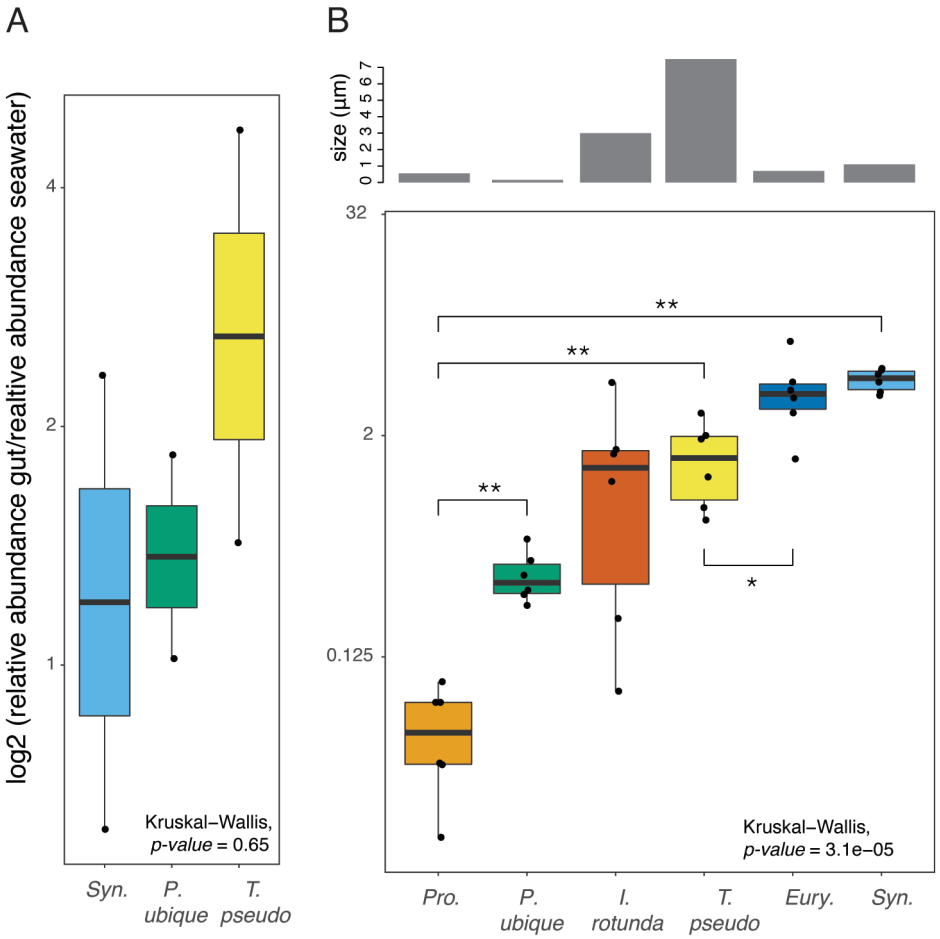

EMI_16334_Figure 5 prey quant and qPCR SECOND REVISED.tif