Hidden interactions in the intertidal rocky shore: variation in pedal mucus microbiota among marine grazers that feed on epilithic biofilm communities

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ABSTRACT

In marine ecosystems, most invertebrates possess diverse microbiomes on their external surfaces, such as those found in the pedal mucus of grazing gastropods and chitons that aids displacement on different surfaces. The microbes are then transported around and placed in contact with free-living microbial communities of micro and other macro-organisms, potentially exchanging species and homogenizing microbial composition and structure among grazer hosts. Here, we characterize the microbiota of the pedal mucus of five distantly related mollusk grazers, quantify differences in microbial community structure, mucus protein and carbohydrate content, and, through a simple laboratory experiment, assess their effects on integrated measures of biofilm abundance. Over 665 Amplicon Sequence Variants (ASVs) were found across grazers, with significant differences in abundance and composition among grazer species and epilithic biofilms. The pulmonate limpet *Siphonaria lessonii* and the periwinkle *Echinolittorina peruviana* shared similar microbiota. The microbiota of the chiton *Chiton granosus*, keyhole limpet *Fissurella crassa*, and scurrinid limpet *Scurria araucana* differed markedly from one another, and form those of the pulmonate limpet and periwinkle. Flavobacteriaceae (Bacteroidia) and Colwelliaceae (Gammaproteobacteria) were the most common among microbial taxa. Microbial strict specialists were found in only one grazer species. The pedal mucus pH was similar among grazers, but carbohydrate and protein concentrations differed significantly. Yet, differences in mucus composition were not reflected in microbial community structure. Only the pedal mucus of *F. crassa* and *S. lessonii* negatively affected the abundance of photosynthetic microorganisms in the biofilm, demonstrating the specificity of the pedal mucus effects on biofilm communities. Thus, the pedal mucus microbiota are distinct among grazer hosts and can affect and interact non-trophically with the epilithic biofilms on which grazers feed, potentially leading to microbial community coalescence mediated by grazer movement. Further studies are needed to unravel the myriad of non-trophic interactions and their reciprocal impacts between macro- and microbial communities.

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INTRODUCTION

‘Out of sight, out of mind’ has been the approach most ecologists, terrestrial and marine, have followed when it comes to understanding the role of species interactions on the functioning of ecosystems and communities, disregarding microscopic organisms as sufficiently ‘isolated’ from their coexisting macroscopic components. Several exceptions to this oversight abound in the literature (Wahl et al., 2012; Rosenberg & Zilber-Rosenberg, 2016), indeed. The discovery that macroscopic organisms host unique and diverse assemblages of microorganisms, the microbiomes, transformed our understanding of animal biology, ecology, and evolution (Lafferty et al., 2008; McFall-Ngai et al., 2013) and it certainly accelerated research on the connections and dependencies between macroscopic and microscopic worlds. But these advances have been largely biased towards internal tissue microbiomes, mostly in mammals, and especially in humans (Costello et al., 2012; Tremaroli & Bäckhed, 2012). In marine ecosystems, the study of susceptibility to macroscopic biofouling on artificial surfaces covered by microbial biofilms at sea has been an active focus of research from an applied material science perspective (Salta et al., 2010; Navarrete et al., 2019; Navarrete et al., 2020; Daille et al., 2020; Antunes et al., 2020). Still, in most natural systems, our understanding of the macroscopic and microscopic interactions remains rudimentary, at best.

The intensively studied rocky shore communities represent a model system that can help us disentangle the complex networks of interactions between microbes and the co-occurring macroscopic invertebrates and macroalgae (Hawkins et al., 2008; Kéfi et al., 2015). Pioneering studies on many rocky shores have documented trophic interactions between invertebrates and microbial biofilms (Nicotri, 1977; Underwood, Denley & Moran, 1983; Hill & Hawkins, 1991; Thompson et al., 2000). However, consumption is but one of the diverse types of interactions that can occur between these worlds. Non-trophic interactions are probably as important and diverse, and some of those interactions have no similar counterpart in the macroscopic world (Kéfi et al., 2012). This is the case of microbiomes of macroscopic organisms, which interact with microorganisms found, for instance, in the epilithic biofilms. Interactions between these microscopic communities, mediated by grazers, could produce wholesale exchanges of species at all trophic levels, also known as community coalescence (Rillig et al., 2015), but in this case, occurring frequently and extensively as the grazers move about the shore.

Recent studies have described the microbial species composition and some of the functional roles of microbiomes of marine gastropods and polyplacophorans, including gut ducts (Dudek et al., 2014; Aronson, Zellmer & Goffredi, 2016; Cicala et al., 2018), gill cells (Zbinden et al., 2015), the outer body surface (Fukunaga et al., 2008; Davis et al.,
Pedal mucus, which is essential for animal motility in all mobile gastropods and chitons (Denny, 1980) and is secreted by the pedal gland located inside the front end of the foot (Davies & Hawkins, 1998), is in direct contact with the rock surface and biofilm microbial communities, yet its microbiome has not been studied in detail. As the organism moves about grazing on the shore, some of the onboard microbial communities of the foot are placed in direct contact with the different components (e.g., species) of biofilm communities found on the rock surface, potentially exchanging species and interacting in ways that could modulate the grazer pedal mucus microbiomes as well as biofilm diversity, composition, and functional attributes. Mollusk pedal mucus is composed of about 96% of water and the ∼4% has different proportion of proteins, carbohydrates, lipids glycoproteins (Denny, 1980; Davies, Hawkins & Jones, 1990), and mineral salts (Shashoua & Kwart, 1959).

Pedal mucus can have a negative effect on biofilms, like antibacterial activity, described in the mucus trail of the predatory whelk Achatina fulica over both Gram-negative and Gram-positive cultures (Iguchi, Aikawa & Matsumoto, 1982). Similarly, the pedal mucus of the grazing pulmonate limpet, Siphonaria pectinata, contains the pectinate antibiotic, which acts against Gram-positive bacteria like Staphylococcus aureus, Bacillus subtilus, and fungi like Candida albicans, and Saccharomyces cerevisiae on cultured experiments (Biskupiak & Ireland, 1983). It has been suggested that antibacterial activity in the mucus is probably associated with the protein and polypeptide concentration, not the carbohydrates, due to higher presences of enzymes and/or pyrone derivative in the pedal mucus (Iguchi, Aikawa & Matsumoto, 1982; Biskupiak & Ireland, 1983).

In contrast, it has been shown that microorganisms and early stages of macroalgae can settle and grow faster on the mucus trail of intertidal grazers (Littorina peruviana, Tegula atra, Siphonaria lessonii, and Collisella sp.) than on clean rock surfaces along the central coast of Chile (Santelices & Bobadilla, 1996). Similar positive effects on microalgal growth were described for the pedal mucus of the limpets Lottia gigantea, Collisela digitalis, and Collisela scabra on the Pacific shore of North America (Connor, 1986). Increased colonization of heterotrophic microorganisms was stimulated by the pedal mucus of the turbinid snail Monodonta turbinata, suggesting the mucus provides organic enrichment for microbial growth (Herndl & Peduzzi, 1989). That is also the case for the pedal mucus of the small abalone, Haliotis diversicolor, which stimulates the growth of bacteria Escherichia coli and Staphylococcus epidermidis (Guo et al., 2009). Biochemical analyses suggest that the ability of mucous trails to trap microalgae adhesively and to stimulate microalgae growth is correlated with carbohydrate content (Connor, 1986).

Thus, the pedal mucus of mobile mollusks can affect epilithic microbial communities. The effects can be positive or negative on some microorganisms, which may be related to the mucus chemical (protein/carbohydrate concentration) or microbiome composition. Identifying a core microbiota, common members to two or more microbial assemblages associated with a habitat (Turnbaugh et al., 2007; Hamady & Knight, 2009; Shade & Handelsman, 2012) is the first step in defining pedal mucus communities, understanding
responses to perturbation, and the components that are resilient and persistent across microbial assemblages (Shade & Handelsman, 2012).

The wave-exposed rocky shores of central Chile are characterized by a diverse assemblage of molluskan grazers that belong to different orders and families and co-occur closely (Santelices, Vásquez & Meneses, 1986; Rivadeneira, Fernández & Navarrete, 2002; Aguilera & Navarrete, 2011; Aguilera & Navarrete, 2012). Although their impacts on macroalgal communities and ecological succession can be quite different (Aguilera & Navarrete, 2012; Aguilera, Navarrete & Broitman, 2013; Aguilera et al., 2020), they overlap in their diets (Santelices, Vásquez & Meneses, 1986; Camus, 2008; Camus, Arancibia & Ávila Thieme, 2013) and all feed regularly on microbial biofilms (Camus et al., 2009; Aguilera, Navarrete & Broitman, 2013; Kéfi et al., 2015). In this model ecosystem, we characterized the pedal mucus microbiota of five common, but distantly related, intertidal mollusks of central Chile as a first step in understanding potential non-trophic interactions between grazers and epilithic biofilms. Using an array of molecular techniques and replicated experiments, we evaluated two general hypotheses: (1) that because all these grazers co-occur on the same wave-exposed rocky shore habitat and consume microbial biofilm, they all will share similar microbial communities in the microbiota of the pedal mucus, with same core microbial groups dominating both epilithic biofilms and grazer microbiota, (2) since the intertidal grazers species may have differences in the pedal mucus content of protein/carbohydrates, these will correlate with differences in their pedal mucus microbiota composition, and in the effects of pedal mucus on the abundance of photosynthetic biofilm.

**MATERIALS & METHODS**

**Grazer assemblage**

We characterized the intertidal mollusk grazer assemblage of the wave exposed shores of Las Cruces, central Chile (33°30′S, 71°38′W) during December 2017 and January 2018. Gently inclined (~40°) rocky platforms were chosen inside the marine reserve of the Estación Costera de Investigaciones Marinas, Pontificia Universidad Católica de Chile, from which fishermen have been excluded since 1982 (Castilla & Durán, 1985; Castilla, 1999; Navarrete, Gelcich & Castilla, 2010) and similarly exposed platforms outside the reserve, roughly 145.9 m to the south, where fishers collect some of the mollusk species. At the high, mid, and low intertidal zones (following Castilla, 1981; Fernández et al., 2000), we haphazardly laid down ten 50 × 50 cm quadrats (0.25 m²) along a 15 m long transect parallel to the shoreline. All grazers inside quadrats were measured (maximum length). We estimated the biomass (g m⁻²) of each species through the wet-weight average per m⁻² (see data in Figshare: https://doi.org/10.6084/m9.figshare.15113490.v2).

We chose five of the most abundant species in terms of total biomass (Fig. 1): one Polyplacophoran, the chiton *Chiton* granosus Fremby, 1828 (Family Chitonidae), and four Gastropods, the Littorinid *Echinolittorina peruviana* Lamarck, 1822 (Family Littorinidae), the keyhole limpet *Fissurella crassa* Lamarck, 1822 (Family Fissurellidae), the scurrinid limpet *Scurria araucana* d’Orbigny, 1839 (Family Lottiidae), and the pulmonate limpet *Siphonaria lessonii* Blainville, 1827 (Family Siphonariidae) (Espoz et al., 2004; Aguilera,
Figure 1  Grazer assemblage biomass at the intertidal rocky shore of central Chile. (A) Grazer assemblage biomass (g m$^{-2}$) at the intertidal rocky shore of central Chile. (a) High, (b) Middle, and (c) Low intertidal zones (mean ± SE). (B) Species selected for analysis are highlighted in colors: (blue) Chiton granosus, (orange) Echinolittorina peruviana, (purple) Fissurella crassa, (pink) Scurria araucana, (green) Siphonaria lessonii. Scale bar is shown for each species.

Navarrete & Broitman, 2013; Camus, Arancibia & Ávila Thieme, 2013). These five species are evolutionarily distantly related and can co-occur closely in wave exposed platforms and other intertidal microhabitats. They are classified as ecological trophic omnivores, scraping the rock surfaces, removing spores, macroalgae seedlings, epiphytes, microorganisms (periphyton and epilithic biofilm) and also newly established invertebrates (Santelices, Vásquez & Meneses, 1986; Aguilera & Navarrete, 2007; Aguilera & Navarrete, 2011; Aguilera & Navarrete, 2012; Navarrete & Broitman, 2013). These five species are evolutionarily distantly related and can co-occur closely in wave exposed platforms and other intertidal microhabitats. They are classified as ecological trophic omnivores, scraping the rock surfaces, removing spores, macroalgae seedlings, epiphytes, microorganisms (periphyton and epilithic biofilm) and also newly established invertebrates (Santelices, Vásquez & Meneses, 1986; Aguilera & Navarrete, 2007; Aguilera & Navarrete, 2011; Aguilera & Navarrete, 2012; Camus, 2008).

Pedal mucus microbiota analyses

The study was conducted at the Estación Costera de Investigaciones Marinas (ECIM) of Pontificia Universidad Católica de Chile (PUC), at Las Cruces, Valparaíso, Chile: 33°30′S, 71°38′. Field study approval number ID Protocol: 170829006, by the Comité Institucional de Seguridad en Investigación of the Pontificia Universidad Católica de Chile. We collected ten animals of each species from gently sloping wave-exposed platforms nearby the marine reserve of Las Cruces, focusing on the intertidal zone they were more abundant (Fig. 1). All animals were collected during nocturnal low tides to prevent foot damage (Aguilera &
Navarrete, 2011; Aguilera & Navarrete, 2012), and the shell and foot area were measured (Supplement 1). Collected individuals were brought to the laboratory in coolers and then placed to acclimatize for a week in separate aquaria to reduce animal stress, with the same source of circulating seawater and constant aeration. There, grazers could only feed on epilithic biofilm that was provided in the same manner to all individuals. To minimize contamination from grazers’ feces, two days before the experiment, individuals were “cleaned” by placing them in an aquarium with constant aeration and 0.2 µm filtered seawater (taken from the same location). The two day period was long enough to reduce feces during the experiment and short enough to avoid locomotory and metabolic adverse effects (Calow, 1974), because on the third day animals reduced their motility (C Arboleda-Baena, 2019, per. obs.). The sterile water was replaced every 2–6 h to minimize ammonium concentration and prevent biofilms formation associated with animals’ feces. We expect that this cleaning period also helped remove microorganisms that are incidental on the animal foot and do not maintain populations in the pedal mucus. The motility and behavior of the animals were monitored throughout the acclimation period. Seawater temperature was maintained at 13 °C ± 2 °C, which was the average SST during the time of experiments. To obtain samples of the pedal mucus microbiota, five grazers of each species were chosen randomly and placed in individual clean aquaria (14.3 ×14.3 × 12.5 cm) with 400 ml filtered seawater (0.22 µm filtered and taken from the same location). Five treatments (a-e) with 5 replicates each were applied for mollusk pedal mucus collection. The experimental unit had cover glass slides of 75 × 25 × 1.5 mm previously autoclaved and placed in the bottom of the aquaria. In these individual arenas we included one individual of (a) Chiton granosus, (b) Echinolittorina peruviana, (c) Fissurella crassa, (d) Scurria araucana, or (e) Siphonaria lessonii. We used as control five aquaria with natural epilithic biofilms grown on granitic rock subjected to the same source of circulating seawater where the grazers were acclimatized. Rocks were collected from the field, cut into coupons of 3 × 8 × 2 cm with a COCH Bridge saw machine that prevented overheating and potential mineral modification, and cleaned by deionized water, dried, maintained at room temperature before introducing them in experimental aquaria. The biofilm growing on the rock surface without grazers was used as a reference for free living epilithic biofilms against which we compare grazer microbiota. Naturally growing biofilms vary greatly in composition and structure depending on successional stage and a suite of microclimatic and environmental conditions (Dang & Lovell, 2016). We therefore opted to use a single common reference for epilithic biofilm from the same source of circulating seawater where the grazers were acclimatized, i.e., for all grazers microbiota.

Treatments were randomly assigned to the 30 experimental units (five replicates per treatment). Every three hours, temperature was checked, and feces were removed. The experiment lasted 24 h, and then the animals were carefully removed. Within 24 h, rocks and cover glass slides of all treatments were sonicated separately (Morris, Monier & Jacques, 1998; Bjerkan, Witsø & Bergh, 2009), filtered through 0.22 µm pore filters of hydrophilic polyether sulfone (Merck), and preserved in liquid nitrogen at −196 °C for later molecular analyses. During the microbiota analyses, we lost one sample of each of the following treatments due to a poor-quality Illumina sequencing run: C. granosus, E.
peruviana, S. lessonii, and epilithic biofilm control (Supplement 1). The largest mean foot area of individuals used in mucus collection was 23.05 ± 3.46 cm² for F. crassa and the lowest value was for S. lessonii with 0.15 ± 0.02 cm². The mean rock area analyzed was 24.84 ± 1.89 cm² (Supplement 1).

**DNA extraction and 16S rRNA-gene sequencing**

DNA extraction from filters was conducted with the Phenol-Chloroform method (Fuhrman et al., 1988). DNA concentration was measured with the Qubit HS dsDNA Assay kit in a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) according to manufacture protocols. The V4-V5 region of the 16S rRNA gene was amplified with the primers 515FB: GTGYCAGCMGCCGCGGTAA and 926R: CCGYCAATTYMTTTRAGTTT (Quince et al., 2011; Parada, Needham & Fuhrman, 2016). Amplicons were sequenced in a MiSeq Illumina platform (2 × 300 bp). Both PCR and sequencing were done at the Dalhousie University CGEB-IMR (https://imr.bio/). Sequence data were deposited at the European Nucleotide Archive (ENA) database under accession number PRJEB41739.

**Community analyses**

Amplicon reads were analyzed using the DADA2 pipeline (Callahan et al., 2016; Lee, 2019) to characterize Amplicon Sequence Variants (ASVs) (Callahan, McMurdie & Holmes, 2017) that were used as a proxy of microbial species or Operational Taxonomic Units (OTUs). All graphics and statistical analyses were carried out in R with RStudio interface (Racine, 2012; R Core Team, 2013). Most community ecology analyses were carried out using the packages vegan v2.5-6 (Oksanen et al., 2015) and phyloseq v1.30.0 (McMurdie & Holmes, 2013). Two rarefaction curves were generated, the first one with a fixed sampling effort of 1,869 reads per sample due to the size of the smallest dataset from one replicate of the epilithic biofilm control (Supplement 1 Fig. S1). This dataset was used to compare between grazer microbiota and microbial communities of epilithic biofilms. The second rarefaction curves, with a fixed sampling effort of 10,599 reads per sample due to the size of the smallest dataset from one replicate of F. crassa microbiota, was used to compare between grazer microbiotas (Supplement 1, Fig. S2). To examine the microbial beta diversity of the grazer species (i.e., pedal mucus) and epilithic biofilm, we used non-metric multidimensional scaling (NMDS) ordination, based on Bray–Curtis and Jaccard dissimilarities. To test for statistically significant differences in composition among the microbiota, we conducted a permutational analysis of variance (PERMANOVA) (Anderson & Walsh, 2013). To determine which treatment differed from others, we conducted pairwise post-hoc tests with False Discovery Rate (FDR) correction (Benjamini & Hochberg (1995). We performed PERMDISP to test for differences in dispersions between groups (Anderson, 2006).

To compare richness and diversity among treatments we conducted separate one-way ANOVAs on richness and Shannon index, after inspection for normality and homoscedasticity, considering treatment (grazer species and control) as a fixed factor and used Tukey’s post hoc test to establish the pattern of differences.

We defined microbe (ASV) habitat specialists and generalists according to the number of grazer species’ microbiota they inhabit. To reduce the importance of rare ASVs, we
omitted ASVs with abundances below 100 reads (Logares et al., 2013) in the dataset with a fixed sampling effort of 10,599 reads. To quantify specificity to different pedal mucus microbiota, we calculated the Indicator Value (IndVal) (Dufrene & Legendre, 1997) with the labdsv package (Roberts & Roberts, 2016) as IndVal_{ij} = Specificity_{ij} \times Fidelity_{ij} \times 100.

Where the Specificity_{ij} is the proportion of samples of type “j” that contain an ASV “i” and the Fidelity_{ij} is the proportion of the number of reads (abundance) of an ASV “i” that are in a “j” type of samples (Dufrene & Legendre, 1997).

**Pedal mucus protein and carbohydrate concentration**

We conducted protein and carbohydrates analyses by collecting, during nocturnal low tides, seven animals of each species from the same platforms as those collected for experiments. Individuals were brought to the laboratory in coolers and then placed in separate aquaria with circulating seawater and constant aeration for one week. This time period reduces animal stress and allowed animals to acclimate to lab conditions. Then, collection of pedal mucus was done under a laminar flow cabinet (Connor, 1986). Animals were carefully removed from their containers, washed in filtered seawater (0.2 µm pore-size filters), and then placed individually on one inclined sterile glass slides (21 × 7 cm). Seven individuals of each species were placed in equal number of glass slides. Filtered seawater was added to stimulate movement and mucus production as the animal moved across the glass surface. They were removed from de glass after a maximum of 5 min of movement. To confirm the mucus pedal of the grazers was successfully collected, we used two of the glass slides (n = 2) and one of them was stained with Gram method (Beveridge, Lawrence & Murray, 2007) to detect gram-positive or gram-negative bacteria, and the other slide was stained with 0.01% acridine orange to stain DNA and RNA of the mucus microbial community (Supplement 2).

The mucus on the other five replicate glass slides was removed with a sterile scalpel and put it in individual cryovials with filtered seawater. Five cryovials were used to determine the mucus carbohydrate concentration (n = 5) with the phenol-sulfuric acid method (Masuko et al., 2005) and the protein concentration (n = 5) with the Bradford method (Bradford, 1976). Data were log-transformed and, since carbohydrates and protein are correlated, we tested for significant differences among carbohydrate and protein concentration among species using ANOVAs separately (Tukey post hoc test for heterogeneous variances was performed) and MANOVA, considering grazer species as a fixed factor. When a significant overall effect was detected, a Linear Discriminant Analysis (LDA) was used to cluster the different pedal mucus of the grazers by the carbohydrate and protein content.

Finally, to measure pedal mucus pH, we used five additional animals of each species, collected from the same platforms. Animals were brought to the laboratory in coolers and the foot pH was measured with a MQuant® pH test strips (resolution: 1.0 pH unit). These pH data were log-transformed (n = 5) to improve normality and then compared among grazer species using a Welch’s ANOVA because slight heteroscedasticity remained after transformation. We considered grazers species as a fixed factor. Then, a Games-Howell post hoc test for heterogeneous variances was performed.
Pedal mucus effects on integrated measures of epilithic biofilms abundance

To get a preliminary assessment of the effect of pedal mucus on integrated measures of the free-living biofilm community, we conducted a replicated laboratory experiment at ECIM to quantify effects on the abundance of photosynthetic biofilm components. To this end, we collected 4 animals of each species from wave-exposed platforms and brought them to the laboratory as described above. Collection of pedal mucus was conducted as described above.

We cultured epilithic biofilms of the intertidal rocky shore and then we took and placed biofilm inoculum on 24 cover glass slides inside a Polycarbonate cell culture plate of 6-Wells, for one week in K medium (Keller et al., 1987). The experiment consisted of placing the pedal mucus collected from the four individuals of each of the five species, in separate replicated wells with cover glass slides that had been cultured with biofilm. Four control wells received no mucus. Treatments were randomly assigned to the cell culture plates. After one week, we stained the cover glass slides with 200 µL 0.01% acridine orange (Rigler, 1966) for 5 min, and then we removed the biofilm. After 3 min incubation in the dark, the staining solution was removed, and the plate was washed twice with 500 µL of PBS solution. We took five photographs of each cover glass slide under a Fluorescent Carl filter 525/50 nm. Then, from the photographs, we measured the cover of the Zeiss AXIO Scope A1 Microscope using excitation filter (FS38) 470/40 nm and emission photosynthetic epilithic biofilm using the software Image J (Arboleda-Baena et al., 2022).

Covers under different treatments (n = 20) were analyzed with one-way ANOVA with grazer species as a fixed factor. A Tukey post-hoc test was performed to determine the pattern of differences.

RESULTS

Microbiota and epilithic biofilm microbial communities

We obtained 683,494 good-quality sequences from 26 samples. After rarefying to 1,869 reads per sample, due to the size of the smallest dataset from one epilithic biofilm sample, we had a total of 958 ASVs from all treatments (Supplement 1), of which 666 ASVs were found in the pedal mucus microbiota of the five grazers and the others in the epilithic biofilm community. Sequences from more than 17 Phyla were found in all mucus microbiota. Proteobacteria (Alphaproteobacteria and Gammaproteobacteria), Bacteroidetes, and Verrucomicrobia sequences were the most abundant and accounted for more than 90% of the reads (Fig. 2). The pedal mucus microbiota of the grazers C. granosus, F. crassa, and S. araucana were dominated by Bacteroidetes sequences (66.5%, 67.3%, and 49.9% relative abundance, respectively); while the pedal mucus microbiota of E. peruviana and S. lessonii were dominated by Gammaproteobacteria (66.2% and 77.5% of their total reads, respectively), and Epsilonbacteraeota (14.7% and 6.3%, respectively) sequences that were absent in the other three species (Table S1 in Supplement 3). In contrast, in the epilithic biofilm, Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes, Planctomycetes and Verrucomicrobia sequences were dominant (Fig. 2), but the most abundant were
Alphaproteobacteria (60.6% of total reads) and Planctomycetes (19.7% of total reads). The last was absent in the pedal mucus microbiota (Fig. 2; Table S1 in Supplement 3).

All mollusk species had, as the most abundant microorganisms sequences, the Family Flavobacteriaceae followed by the Colwelliaceae (Bacteroidetes and Gammaproteobacteria respectively) (Table S2 in Supplement 3). The microbial communities of all species were different, with low dispersion of composition within *F. crassa* and *C. granosus*, and a large dispersion in *S. araucana* (Fig. 3). There was only high community overlap between the microbiota of *E. peruviana* and *S. lessonii* (Fig. 3). Analyses of the epilithic biofilm showed large and significant differences with the pedal mucus microbiota of all species (Supplement 4). Differences in microbial communities among grazer species and epilithic biofilms were statistically significant with Bray-Curtis and Jaccard distances (PERMANOVA, $df = 5$, $p = 0.0009$), and FDR-adjusted post-hoc tests showed that all species differed from each other, except *E. peruviana* and *S. lessonii* ($p$ adjusted = 0.9, for both distances. Tables S1 and S2 in Supplement 4). We performed the same analyses with the rarefaction level dataset of 10,599 reads and compared the similarity between grazer microbiotas. We obtained the same results with Bray–Curtis and Jaccard distances, differences that were statistically significant between grazers microbiota (PERMANOVA, $df = 5$, $p = 0.0009$), and the FDR-adjusted post-hoc test showed no differences between the microbial communities of *E. peruviana* and *S. lessonii* ($p$ adjusted = 0.9, for both distances. Tables S3 and S4 in Supplement 4). After PERMDISP analysis, we proved, with an ANOVA test, the groups’ dispersions were not different for both rarefaction datasets (Figs. S3 and S4 in Supplement 4).
Figure 3  Microbiota compositional similarity of the most abundant grazers of the intertidal rocky shore and the epilithic biofilm. Non-metric multidimensional scaling (NMDS) ordination plots based on Bray–Curtis distances. The shapes denote the microbiota grazer species surrounded by an ellipse showing the 95% confidence interval: (♦) Chiton granosus, (■) Echinolittorina peruviana, (▲) Fissurella crassa, (▲) Scurria araucana, (●) Siphonaria lessonii, and (■) Epilithic biofilm. Stress = 0.101.

We found significant differences in microbiota richness (mean + SE) among treatments (Fig. S1A in Supplement 5, ANOVA, df = 5, p = 0.0001), with similar values among F. crassa (138.6 ± 8.39), C. granosus (165.25 ± 14.97), S. araucana (130.6 ± 21.09) and epilithic biofilms (137.75 ± 29.37), and significantly lower richness in E. peruviana (67.25 ± 6.42) and S. lessonii (66.25 ± 6.54) (Table S1 in Supplement 6; Tukey post hoc tests). The Shannon diversity index (mean + SE) also showed significant differences among treatments (Supplement 5 Fig. S1B, ANOVA, df = 5, p = 0.0002), epilithic biofilm (4.22 ± 0.14), C. granosus (3.63 ± 0.28), E. peruviana (2.77 ± 0.12), F. crassa (3.83 ± 0.06), S. araucana (3.20 ± 0.27), and S. lessonii (2.71 ± 0.17). However, it was not possible to resolve a clear pattern of difference between species pairs using the Tukey post-hoc test due to a lack of power (Table S2 in Supplement 6). Richness changed with a rarefaction level of 10,599 reads (Supplement 1 and Fig. S2 in Supplement 5). Nevertheless, differences between richness and diversity treatments maintain the same pattern observed for rarefaction level <2000 reads (See Fig. S2 in Supplement 5; Tables S1–S4 in Supplement 6).

After rarefying to 10,599 reads per sample, due to the size of the smallest dataset from one F. crassa sample, we had a total of 1,205 ASVs from all pedal mucus microbiota treatments (Supplement 1). The pedal mucus microbiome of C. granosus displayed an average of 236 ASVs, 107 ASVs for E. peruviana, 189 ASVs for F. crassa, 237 ASVs for S. araucana, and 112 ASVs for S. lessonii (Supplement 1). Our analyses of specificity...
and fidelity at the microbial family level, which reduced the number of ASVs to 123 taxa, showed that the pedal mucus microbiota of *C. granosus*, *E. peruviana*, *F. crassa*, and *S. araucana* were composed mostly by Flavobacteriaceae sequences. *C. granosus* also has habitat specialists from Bacteroidetes (Crocinitomicaceae, Cryomorphaceae, Cyclobacteriaceae, and Saprospiraceae), Alphaproteobacteria (Rhodobacteraceae and Phyllobacteriaceae), Gammaproteobacteria (Alteromonadaceae) and Verrucomicrobia (Rubritaleaceae) sequences. In turn, *E. peruviana* has habitat specialist ASVs from Epsilonbacteraeota (Arrobacteraceae), Gammaproteobacteria (Colwelliaceae and Nitrincolaceae) and Verrucomicrobia (Rubritaleaceae) sequences. *F. crassa* has habitat specialists from Bacteroidetes (Crocinitomicaceae and Cyclobacteriaceae), Alphaproteobacteria (Rhodobacteraceae), Gammaproteobacteria (Nitrincolaceae, Cardiobacteriaceae, Pseudoalteromonadaceae, Vibrionaceae, and Shewanellaceae), Verrucomicrobia (Rubritaleaceae), Deltaproteobacteria (Bacteriovoraceae) and Spirochaetes (Spirochaetaceae) sequences. *S. araucana* has habitat specialists from Alphaproteobacteria (Rhodobacteraceae) and Gammaproteobacteria (Nitrincolaceae) sequences. In the case of *S. lessonii*, the most abundant habitat specialist ASVs were Alphaproteobacteria (Rhodobacteraceae), but it also has ASVs from Gammaproteobacteria (Alteromonadaceae, Cellvibrionaceae and Marinomonadaceae) sequences (Fig. 4; Supplement 7).

**Pedal mucus protein and carbohydrate concentration**

The method developed to obtain pedal mucus from live individuals was successful in all cases (Supplement 7). We found statistical differences in carbohydrate content among mollusk pedal mucus (Fig. 5A, and Table S1 in Supplement 8, ANOVA, df = 4, p = 0.0004). The carbohydrate content values µg/ml (mean ± SE) in descending order were *F. crassa* (41.96 ± 8.59), *C. granosus* (11.49 ± 4.50), *E. peruviana* (6.28 ± 12.21), *S. lessonii* (2.69 ± 11.29) and *S. araucana* (2.65 ± 2.04). The protein content also varied among pedal mucus grazers (Fig. 5B, and Table S2 Supplement 8, ANOVA, df = 4, p = 0.006). The protein content values µg/ml (log mean ± SE,) were *F. crassa* (1.61 ± 0.4), *S. lessonii* (0.94 ± 0.35), *E. peruviana* (0.51 ± 0.21), *C. granosus* (0.27 ± 0.27), and *S. araucana* (0 ± 0). Both mean carbohydrate and protein concentration varied significantly among grazer species (Fig. 5A and Fig. 5B, MANOVA, df = 4, p = 0.018). The bivariate plot of carbohydrate and protein content showed the pedal mucus of *F. crassa* had a much higher concentration of both components than that of all other species. In contrast, *S. araucana* had the lowest, with nearly nil protein content (Fig. 5D). To cluster the different pedal mucus of the grazers by the carbohydrate and protein content, we performed an LDA. This showed slightly different patterns with the limpets *F. crassa* and *S. lessonii* clustering together and on opposite bivariate ends than *S. araucana* (Supplement 9).

The mean pedal mucus pH ranged from 8.2 to 9.0 among species, with little variability among individuals within species or among species (Fig. 5C). In some cases (*C. granosus*, *E. peruviana*) no variation in pH was detected among individuals.
Pedal mucus effects on epilithic biofilms

The pedal mucus of both the keyhole limpet *F. crassa* and the pulmonated *S. lessonii* significantly reduced epilithic cover with respect to controls (ANOVA and the Tukey post-hoc test, $df = 5$, $p < 0.001$ and $p = 0.02$, respectively), with the effect of the keyhole limpet significantly higher than that of *S. lessonii* (Fig. 6). The mucus of all other species had no effect over epilithic biofilm (Fig. 6).

**DISCUSSION**

Microbiota and epilithic biofilm microbial communities

We found that the pedal mucus microbiota of all species tested were different from that of the epilithic biofilms, and that they also differed among species. The most similar microbiota were those between the pulmonate limpet *S. lessonii* and that of the periwinkle *E. peruviana*, which both tend to occupy the upper intertidal shore (Santelices, 1990; Hidalgo et al., 2008). In contrast, the microbiota of the chiton *C. granosus*, keyhole limpet *F. crassa*, and scurrinid limpet *S. araucana* differed markedly from all grazers. Thus, we reject the hypothesis that the same core microbial groups dominating the epilithic biofilms are also dominant in grazer microbiota. Also, we reject that grazers share similar microbial communities in their external microbiota because they all co-occur on the same wave-exposed rocky shore habitat and consume biofilms. Our results are similar with previous studies which found that some invertebrate microbiota were different from...
Figure 5  Pedal mucus Carbohydrates/Proteins content and pH. Pedal mucus characteristics of *Chiton granosus*, *Echinolittorina peruviana*, *Fissurella crassa*, *Scurria araucana*, and *Siphonaria lessonii*.  (A) Carbohydrate content of pedal mucus  (B) Protein content of pedal mucus  (C) Pedal mucus pH (mean + SE). Different letters above bars indicate significant differences with a posteriori Tukey tests at the experiment-wise error rate = 0.05.  (D) Correlation between carbohydrates and protein content of pedal mucus (bars indicated the SE).

Characterization of host-microbiota is challenging, as it is too easy to include the pool of species present in the natural environment (e.g., seawater) at the time animals are collected as host-microbiota. It is important to identify and remove those species in the surrounding environment when animals are collected from those habitat specialists in the animal’s body or the pedal mucus, which requires laboratory acclimations and sterile seawater. However, further research with different approaches is needed to understand and capture the variations of the complete natural microbial communities.

In our study, *E. peruviana* and *S. lessonii*, those with the most similar microbiota co-occur with all other grazer species on wave-exposed rocky platforms (*Broitman et al., 2001; Rivadeneira, Fernández & Navarrete, 2002*). Nevertheless, they are found mostly in the upper intertidal zone more frequently than the other grazer species (*Fig. 1*) (*Otaíza & Santelices, 1985; Santelices, Vásquez & Meneses, 1986; Aguilera & Navarrete, 2007*). Harsh...
Figure 6  Pedal mucus effect over photosynthetic biofilm cover percentage. Fluorescence microscopy of pedal mucus effect on photosynthetic biofilm cover percentage. Treatments (A) Control with no pedal mucus, (B) Chiton granosus, (C) Echinolittorina peruviana, (D) Fissurella crassa, (E) Scurria araucana, and (F) Siphonaria lessonii. Different letters above bars indicate significant differences (p < 0.05) among treatments (Tuckey test after one-way ANOVA). Photograph selected from a set of 20 replicates. Photography by Clara Arboleda-Baena.

physical conditions encountered in the upper shore, such as temperature (Wethey, 1983; Williams & Morritt, 1995; Finke, Navarrete & Bozinovic, 2007; Szathmary, Helmuth & Wethey, 2009), solar radiation (Santelices, 1990; Huovinen & Gómez, 2011), and desiccation stresses (Evans, 1947; Stephenson & Stephenson, 1961; Lewis, 1964; Castilla, 1981; Santelices, 1990; Harley & Helmuth, 2003; Flores, Cienfuegos & Navarrete, 2019) may influence the composition of the pedal mucus microbiota (higher selection pressure) of these two species, making them more similar. The higher relative abundances of Arcobacteraceae, Alteromonadaceae, Colwelliaceae, Marinomonadaceae, and Saccharospirillaceae (Table S2 in Supplement 3) in the pedal mucus microbiota of E. peruviana and S. lessonii is not closely related to the environmental factors mentioned above, but further studies should be conducted to evaluate the functional characteristics of these ASVs in the grazers’ pedal
mucus. In turn, the less stressful conditions (i.e., lower selection pressure) in the lower shore may permit microbiota to differ more widely among grazer species (Arboleda-Baena et al., 2021). A larger variety of species from different shore levels must be investigated to separate effects dependent upon habitat from species-specific effects.

All mollusk species had Flavobacteriaceae followed by the Colwelliaceae (Bacteroidia and Gammaproteobacteria respectively) as the most abundant microorganism sequences (Table S2 in Supplement 3). Bacteroidetes are prone to have a surface-associated lifestyle, supported by the extracellular degradation of complex polymers such as polysaccharides and proteins (Dang & Lovell, 2016), like those found in the pedal mucus. Members of Flavobacteriaceae also have been found in the gastrointestinal tract microbiome of other mollusks, such as the blue-rayed limpet Patella pellucida (Dudek et al., 2014), and are significantly more abundant in the digestive glands of healthy red abalones (Haliothis rufescens) than they are in red abalones with infectious diseases (Villasante et al., 2020). To the best of our knowledge, ours is the first report of the family Colwelliaceae (Alteromonadales) in Gastropoda and Polyplacophora microbiota. We hypothesize that they could be expected in all mollusks’ pedal microbiota because of their capability to hydrolyze organic compounds (Ivanova, Sébastien & Richard, 2004) present in the mucus. Alteromonadales have been described in different marine surfaces (Dang & Lovell, 2016), like macroalgae (Egan et al., 2013; Neu, Allen & Roy, 2019) and metal surfaces exposed at sea (Vasconcelos, 2020).

The “habitat specialists”, ASVs with a higher specificity and fidelity to a specific mollusk microbiota, in C. granosus, E. peruviana, F. crassa, and S. araucana belonged mostly to Bacteroidetes (Flavobacteriaceae). Specifically, C. granosus and F. crassa had taxa from Bacteroidetes (Crocinotomicaceae and Cyclobacteriaceae. C. granosus also has taxa from Cryomorphaceae and Saprospiraceae). Crocinotomicaceae’s (Muñoz, Rosselló-Móraa & Amann, 2016) presence could be explained by the amino acids required for their growth (Bowman, 2020) which are present in the pedal mucus. This is the first description of this bacterial family in the pedal mucus microbiota of Polyplacophora and F. crassa. Moreover, Cyclobacteriaceae (Nedashkovskaya & Ludwig, 2015) is widely distributed in diverse marine habitats such as marine surface water, marine organisms, salty water lagoons, oilfield sediments, and solar salterns (Bhumika et al., 2013). Members of the family were previously described in the microbiota of the blue-rayed limpet Patella pellucida (Dudek et al., 2014). Additionally, Cryomorphaceae (Bowman, Nichols & Gibson, 2003), also presented in C. granosus microbiota, have complex growth requirements necessitating sea-water salts, organic compounds as sole nitrogen sources, yeast extract, and vitamins. Their diversity and particular nutritional preferences suggest a wide range of habitats (Bowman, Nichols & Gibson, 2003; Bowman, 2020), including the chiton pedal mucus. Saprospiraceae (Krieg et al., 2012), for its part, is found in freshwater and/or marine environments, and was previously described in the gut microbiome of the Roswell springsnail (Pyrgulopsis roswellensis) and Koster’s springsnail (futurnia kosteri) (Walters et al., 2022). Their nutritional requirements have not been described yet, and their presence in the pedal mucus requires further research.
Verrucomicrobia ASVs (Rubritaleaceae) (Hedlund, 2015) were found to be habitat specialists in C. granosus, E. peruviana and F. crassa pedal mucus microbiota. The presence of this family in the pedal mucus microbiota may be explained by the oxidation of a wide variety of organic molecules for growth (Hedlund, 2015). This Phylum was previously described in the microbiota of the rough periwinkle (Littorina keenae) (Neu, Allen & Roy, 2019) and in the gut microbiome of the Roswell springsnail (Pyrgulopsis roswellensis) and Koster’s springsnail (Juturnia kosteri) (Walters et al., 2022).

Epsilonproteobacteria (Arcobacteraecea), was only found in E. peruviana pedal mucus, where it reached high abundance. This class was previously described in other gastropod microbiomes (Dudek et al., 2014; Zbinden et al., 2015; Mizutani et al., 2020).

In contrast, C. granosus, F. crassa, S. araucana and S. lessonii had habitat specialist taxa from Alphaproteobacteria (Rhodobacteraceae). Rhodobacteraceae (Garrity, Bell & Lilburn, 2005a), occur in freshwater and marine systems and are frequently associated with biological surfaces (Dang & Lovell, 2016). This family has been previously discovered in gills of the giant abalones (Haliotis gigantea) (Mizutani et al., 2020) and the gut microbiome of the Roswell springsnail (Pyrgulopsis roswellensis) and Koster’s springsnail (Juturnia kosteri) (Walters et al., 2022).

Alphaproteobacteria ASVs (Phyllobacteriaceae) were found to be habitat specialists in C. granosus pedal mucus microbiota. Phyllobacteriaceae has genera present in marine environments, (Brenner et al., 2005; Liu et al., 2016) and members of this family were previously described in the gut microbiome of the Roswell springsnail (Pyrgulopsis roswellensis) and Koster’s springsnail (Juturnia kosteri) (Walters et al., 2022).

Deltaproteobacteria ASVs (Bacteriovoracaceae) and Spirochaetes ASVs (Spirochaetaceae) were found to be habitat specialists in F. crassa pedal mucus microbiota. Bacteriovoracaceae members are aerobic predators of gram-negative bacteria or they can grow saprophytically in a rich nutrient medium (Davidov & Jurkevitch, 2004). Their presence in the pedal mucus microbiota exhibits the complexity of interactions found in this environment. Further research is needed to better understand interactions between microbiota members. Members of Deltaproteobacteria were previously found in the blue-rayed limpet (Patella pellucida) microbiota (Dudek et al., 2014) and the gill cells of the hydrothermal vent gastropod Cyathermia naticoides (Zbinden et al., 2015). However, this is the first report of Bacteriovoracaceae in mollusk microbiota. The presence of Spirochaetaceae microbiota may be explained by carbohydrates or amino acids required for carbon and energy sources present in the pedal mucus (Gupta, Mahmood & Adeolu, 2013), Members of this family have been described in the gills of three abalone species, namely Haliotis discus, H. gigantea, and H. diversicolor (Mizutani et al., 2020).

Pedal mucus microbiota from all grazer species also contained other habitat specialists from Gammaproteobacteria (Alteromonadaceae, Cardiobacteriaceae, Cellvibrionaceae, Colwelliaceae, Marinomonadaceae, Nitrincolaceae, Pseudoalteromonadaceae, Shewanellaceae, and Vibrionaceae) (see Fig. 4). In general, Gammaproteobacteria are surface associated, especially Alteromonadaceae (Dang & Lovell, 2016). Nitrincolaceae (Gammaproteobacteria, Oceanospirillales) (Garrity, Bell & Lilburn, 2005b) has been discovered on the shell surfaces of the California Mussel (Mytilus californianus) in tidepools.
and on emergent benches (Pfister, Meyer & Antonopoulo, 2010), in the gill microbiome of mangrove lucinids (Phacoides pectinatus) (Lim et al., 2019), and the large bivalves (Acesta excavata) in coral reefs on the northeast Atlantic (Jensen et al., 2010). Members of Gammaproteobacteria have been reported as the most plentiful in water and algal samples (Neu, Allen & Roy, 2019) and artificial surfaces (Daille et al., 2020) in the Southeast Pacific. Regarding mollusk microbiomes, members of this class have been found in the digestive gland of red abalones (Haliotis rufescens) (Villasante et al., 2020). They are abundant in the gills of the disk abalone Haliotis discus (Mizutani et al., 2020) and the blue-rayed limpet Patella pellucida (Dudek et al., 2014). This is the first description of some families in the pedal mucus of Gastropoda and Polyplacophora, however, further research is needed to understand their role in the pedal mucus environment.

**Pedal mucus protein and carbohydrate concentration**

According to our results, differences in microbial composition among grazers cannot be explained by differences in the protein/carbohydrate concentrations of their pedal mucus.

The hypothesis that the chemical micro-environment in the pedal mucus determined the composition of the microbial community was therefore rejected. The most similar/different species in terms of microbial community composition were not the most similar/different in mucus composition (compare Figs. 2 and 5). Since grazer species did not exhibit differences in pH, one of the most critical factors in the microbial community structure (Rousk et al., 2010), it is possible that our study of the difference in carbohydrates and proteins does not provide sufficient causal explanations for differences in the microbial community. Thus, a study of protein/carbohydrate identities is needed to understand microbial metabolisms and composition (Martiny, Treseder & Pusch, 2013). Conversely, our study also suggests that interactions within the microbial community and their host may overcome, to some extent, the conditions imposed by variability in mucus composition. Consequently, further studies should be conducted on factors that could affect the microbiome composition, such as aging, development, diet, and grazer reproduction (Apprill, 2017).

**Pedal mucus effects on epilithic biofilms**

Our experimental results show that only the mucus of F. crassa and S. lessonii had significant and negative effects on photosynthetic components of the epilithic biofilm. Since these species tended to have different protein/carbohydrate concentrations than the remainder of the species, especially F crassa, we cannot reject the hypotheses that the effects of pedal mucus on the abundance of photosynthetic biofilm are at least partly related to protein/carbohydrate concentration in the pedal mucus. The protein content is correlated with antibacterial effects, both for gram-negative and gram-positive bacteria (Iguchi, Aikawa & Matsumoto, 1982), while the carbohydrate concentration is not (Connor, 1986; Davies, Hawkins & Jones, 1990). Thus, it appears that the higher protein concentration in F. crassa and S. lessonii pedal mucus (Fig. 5D) could negatively affect the cover of photosynthetic epilithic biofilms. However, further studies must be conducted to test this hypothesis because our study had a small sample size and other variables could also affect the biofilm cover. Additional studies are needed to investigate the role of protein content in
pedal mucus of different grazers, not only on measures of the photosynthetic components of biofilms, but on the entire biofilm community structure through high-throughput sequencing methods.

CONCLUSIONS

The process of evolution in co-occurring micro- and macro-organisms allowed many microorganisms to colonize new habitats in the bodies of macroscopic organisms (McFall-Ngai et al., 2013), such as the pedal mucus of mollusk grazers. In our results, the pedal mucus microbiota and its carbohydrate/protein content showed variability across grazer species and a potential impact on epilithic biofilms, despite these mollusks co-occurring on the same wave-exposed rocky shore habitat and consuming epilithic biofilms. The differences in microbial composition among grazers and the effects of pedal mucus on the abundance of photosynthetic biofilm were not explained by differences in the overall chemical composition (protein/carbohydrate concentration) of the pedal mucus. Consequently, further studies should be conducted to understand the identities of those proteins and carbohydrates and to analyze other factors that could affect the microbiota composition such as aging, development, and grazer reproduction. Our broad description of the main microbial families found in all (generalists) or some (specialist) of the five mollusk grazer microbiota is provided here as a first step towards defining a baseline for microbial communities. However, further studies should be done to define “healthy” or “unhealthy” communities for these grazers. This information would be useful for future assessment of potential responses and alteration due to human-caused perturbations, persistence, and variability of complex microbial assemblages (Shade & Handelsman, 2012; Ribes et al., 2016), and also to identify core microbes associated with other mollusk hosts under normal or perturbed conditions (Shade & Handelsman, 2012), thus providing insight into the ecology and co-evolution of these systems.

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The authors declare there are no competing interests.

Author Contributions
• Clara Arboleda-Baena conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
• Claudia Belén Pareja performed the experiments, prepared figures and/or tables, and approved the final draft.
• Isadora Pla performed the experiments, prepared figures and/or tables, and approved the final draft.
• Ramiro Logares analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
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The sequence data are available at the European Nucleotide Archive (ENA) database: PRJEB41739.

Data Availability
The following information was supplied regarding data availability:
The data is available at Figshare: Available at https://figshare.com/projects/Hidden_interactions_in_the_intertidal_rocky_shore_variation_in_pedal_mucus_microbiomes_among_marine_grazers_that_feed_on_epilithic_biofilm_communities_/119655
Arboleda Baena, Clara María (2021): PedalMucusEffect_PhotosyntheticBiofilmCoverPercentage. figshare. Dataset. https://doi.org/10.6084/m9.figshare.15170586.v1
Arboleda Baena, Clara María (2021): PedalMucus_MANOVA_Carbohydrates/ProteinsContent. figshare. Dataset. https://doi.org/10.6084/m9.figshare.15170523.v1
Arboleda Baena, Clara María (2021): PedalMucus_ProteinsContent. figshare. Dataset. https://doi.org/10.6084/m9.figshare.15170520.v1
Arboleda Baena, Clara María (2021): PedalMucus_CarbohydratesContent. figshare. Dataset. https://doi.org/10.6084/m9.figshare.15170514.v1.
Arboleda Baena, Clara María (2021): GrazerAssemblageBiomass.csv. figshare. Dataset. https://doi.org/10.6084/m9.figshare.15113490.v2.
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REFERENCES

Aguilera MA, Navarrete SA. 2007. Effects of Chiton granosus (Frembly, 1827) and other molluscan grazers on algal succession in wave exposed mid-intertidal rocky shores of central Chile. *Journal of Experimental Marine Biology and Ecology* **349**:84–98 DOI 10.1016/j.jembe.2007.05.002.

Aguilera M, Navarrete S. 2011. Distribution and activity patterns in an intertidal grazer assemblage: influence of temporal and spatial organization on interspecific associations. *Marine Ecology Progress Series* **431**:119–136 DOI 10.3354/meps09100.

Aguilera MA, Navarrete SA. 2012. Functional identity and functional structure change through succession in a rocky intertidal marine herbivore assemblage. *Ecology* **93**:75–89 DOI 10.1890/11-0434.1.

Aguilera M, Navarrete S, Broitman BR. 2013. Differential effects of grazer species on periphyton of a temperate rocky shore. *Marine Ecology Progress Series* **484**:63–78 DOI 10.3354/meps10297.

Aguilera MA, Valdivia N, Broitman BR, Jenkins SR, Navarrete SA. 2020. Novel co-occurrence of functionally redundant consumers induced by range expansion alters community structure. *Ecology* **101**:e03150 DOI 10.1002/ecy.3150.

Anderson MJ. 2006. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**:245–253 DOI 10.1111/j.1541-0420.2005.00440.x.
Anderson MJ, Walsh DCI. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecological Monographs* **83**:557–574 DOI 10.1890/12-2010.1.

Antunes JT, Sousa AGG, Azevedo J, Rego A, Leão PN, Vasconcelos V. 2020. Distinct temporal succession of bacterial communities in early marine biofilms in a Portuguese Atlantic Port. *Frontiers in Microbiology* **11**:1938 DOI 10.3389/fmicb.2020.01938.

Apprill A. 2017. Marine animal microbiomes: toward understanding host–microbiome interactions in a changing ocean. *Frontiers in Marine Science* **4**:222 DOI 10.3389/fmars.2017.00222.

Arboleda-Baena CM, Freilich MA, Pareja CB, Logares R, Dela Iglesia R, Navarrete SA. 2021. Microbial communities network structure across strong environmental gradients: how do they compare to macroorganisms? *biorxiv* DOI 10.1101/2021.06.08.445284.

Arboleda-Baena CM, Pareja C, Pla I, Logares R, Dela Iglesia R, Navarrete SA. 2022. Mollusk pedal mucus effects on epilithic biofilms DOI 10.17504/protocols.io.5qpvoy3bdg4o/v2.

Aronson HS, Zellmer AJ, Goffredi SK. 2016. The specific and exclusive microbiome of the deep-sea bone-eating snail, *Rubyspira osteovora*. *FEMS Microbiology Ecology* **93**:fiw250 DOI 10.1093/femsec/fiw250.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)* **57**:289–300 DOI 10.1111/j.2517-6161.1995.tb02031.x.

Beveridge TJ, Lawrence JR, Murray RG. 2007. Sampling and staining for light microscopy. In: *Methods for general and molecular microbiology*. 3rd edn. Washington, D. C.: American Society for Microbiology Press, 19–33.

Bhumika V, Srinivas TNR, Ravinder K, Anil Kumar P. 2013. Mariniradius saccharolyticus gen, nov. sp. nov. a member of the family Cyclobacteriaceae isolated from marine aquaculture pond water, and emended descriptions of the genus Aquiflexum and Aquiflexum balticum. *International Journal of Systematic and Evolutionary Microbiology* **63**:2088–2094 DOI 10.1099/ijs.0.043919-0.

Biskupiak JE, Ireland CM. 1983. Pectinatone, a new antibiotic from the mollusc *Siphonaria pectinata*. *Tetrahedron Letters* **24**:3055–3058 DOI 10.1016/S0040-4039(00)88093-4.

Bjerkan G, Witosø E, Bergh K. 2009. Sonication is superior to scraping for retrieval of bacteria in biofilm on titanium and steel surfaces in vitro. *Acta Orthopaedica* **80**:245–250 DOI 10.3109/17453670902947457.

Bowman JP. 2020. Out from the shadows—resolution of the taxonomy of the family cryomorphaceae. *Frontiers in Microbiology* **11**:12 DOI 10.3389/fmicb.2020.00012.

Bowman JP, Nichols CM, Gibson JAE. 2003. Algoriphagus ratkowskyi gen, nov. sp. nov. Brumimicrobiurn glaciale gen. nov. sp. nov. Cryomorpha ignava gen. nov. sp. nov. and Crocinitorium catalasitica gen. nov. sp. nov. novel flavobacteria isolated
from various polar habitats. *International Journal of Systematic and Evolutionary Microbiology* 53:1343–1355 DOI 10.1099/ijs.0.02553-0.

**Bradford MM. 1976.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248–254 DOI 10.1016/0003-2697(76)90527-3.

**Brenner DJ, Krieg NR, Staley JT, Garrity G. 2005.** Bergey’s manual® of systematic bacteriology: volume two the proteobacteria part c the alpha-, beta-, delta-, and epsilonproteobacteria. New York: Springer.

**Broitman B, Navarrete S, Smith F, Gaines S. 2001.** Geographic variation of southeastern Pacific intertidal communities. *Marine Ecology Progress Series* 224:21–34 DOI 10.3354/meps224021.

**Burgsdorf I, Erwin PM, López Legentil S, Cerrano C, Haber M, Frenk S, Steindler L. 2014.** Biogeography rather than association with cyanobacteria structures symbiotic microbial communities in the marine sponge *Petrosia ficiformis*. *Frontiers in Microbiology* 5:529 DOI 10.3389/fmicb.2014.00529.

**Callahan BJ, McMurdie PJ, Holmes SP. 2017.** Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal* 11:2639–2643 DOI 10.1038/ismej.2017.119.

**Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016.** DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* 13:581–583 DOI 10.1038/nmeth.3869.

**Calow P. 1974.** Some observations on locomotory strategies and their metabolic effects in two species of freshwater gastropods, *ancylus fluviatilis* miull. and *planorbis contortus* linn. *Oecologia* 16:149–161 DOI 10.1007/BF00345579.

**Camus PA. 2008.** Diversidad, distribución y abundancia de especies en ensambles intermareales rocosos. *Revista de Biología Marina y Oceanografía* 43:615–627 DOI 10.4067/S0718-19572008000300021.

**Camus PA, Arancibia PA, Ávila Thieme I. 2013.** A trophic characterization of intertidal consumers on Chilean rocky shores. *Revista de biología marina y oceanografía* 48:431–450 DOI 10.4067/S0718-19572013000300003.

**Camus P, Cid Y, Cisterna L, Cáceres C. 2009.** Consumption and digestion of animal food by rocky intertidal herbivores: an evaluation of digestive flexibility and omnivory in three grazing species. *Latin American Journal of Aquatic Research* 37:191–197 DOI 10.3856/vol37-issue2-fulltext-6.

**Castilla JC. 1981.** Perspectivas de investigación en estructura y dinámica de comunidades intermareales rocosas de Chile central, 2: Depredadores de alto nivel trópico. *Medio Ambiente* 5:190–215.

**Castilla JC. 1999.** Coastal marine communities: trends and perspectives from human-exclusion experiments. *Trends in Ecology & Evolution* 14:280–283 DOI 10.1016/S0169-5347(99)01602-X.

**Castilla JC, Durán LR. 1985.** Human exclusion from the rocky intertidal zone of Central Chile: the effects on concholepas concholepas (gastropoda). *Oikos* 45:391 DOI 10.2307/3565575.
Cicala F, Cisterna-Céliz JA, Moore JD, Rocha-Olivares A. 2018. Structure, dynamics and predicted functional role of the gut microbiota of the blue (*Haliotis fulgens*) and yellow (*H. corrugata*) abalone from Baja California Sur, Mexico. *PeerJ* 6:e5830 DOI 10.7717/peerj.5830.

Connor VM. 1986. The use of mucous trails by intertidal limpets to enhance food resources. *The Biological Bulletin* 171:548–564 DOI 10.2307/1541623.

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. 2012. Toward an understanding of the human microbiome. *Science* 336(6086):1255–1262 DOI 10.1126/science.1224203.

Daille LK, Aguirre J, Fischer D, Galarce C, Armijo F, Pizarro GE, Walczak M, Dela Iglesia R, Vargas IT. 2020. Effect of tidal cycles on bacterial biofilm formation and biocorrosion of stainless steel AISI 316L. *Journal of Marine Science and Engineering* 8:124 DOI 10.3390/jmse8020124.

Dang H, Lovell CR. 2016. Microbial surface colonization and biofilm development in marine environments. *Microbiology and Molecular Biology Reviews* 80:91–138 DOI 10.1128/MMBR.00037-15.

Davidov Y, Jurkevitch E. 2004. Diversity and evolution of Bdellovibrio-and-like organisms (BALOs), reclassification of Bacteriovorax starrii as Peredibacter starrii gen. nov. comb. nov. and description of the Bacteriovorax–peredibacter clade as Bacteriovoraceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology* 54(Pt 5):1439–1452 DOI 10.1099/ijs.0.02978-0.

Davies MS, Hawkins SJ. 1998. Mucus from marine molluscs. *Advances in Marine Biology* 34:1–71 DOI 10.1016/S0065-2881(08)60210-2.

Davies MS, Hawkins SJ, Jones HD. 1990. Mucus production and physiological energetics in *Patella vulgata* L. *Journal of Molluscan Studies* 56:499–503 DOI 10.1093/mollus/56.4.499.

Davis J, Fricke WF, Hamann MT, Esquenazi E, Dorrestein PC, Hill RT. 2013. Characterization of the bacterial community of the chemically defended Hawaiian sacoglossan elysia rufescens. *Applied and Environmental Microbiology* 79:7073–7081 DOI 10.1128/AEM.01568-13.

Denny M. 1980. The role of gastropod pedal mucus in locomotion. *Nature* 285:160–161 DOI 10.1038/285160a0.

Dudek M, Adams J, Swain M, Hegarty M, Huws S, Gallagher J. 2014. Metaphyllogenomic and potential functionality of the limpet patella pellucida's gastrointestinal tract microbiome. *International Journal of Molecular Sciences* 15:18819–18839 DOI 10.3390/ijms151018819.

Dufrene M, Legendre P. 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecological Monographs* 67:345 DOI 10.2307/2963459.

Egan S, Harder T, Burke C, Steinberg P, Kjelleberg S, Thomas T. 2013. The seaweed holobiont: understanding seaweedbacteria interactions. *FEMS Microbiology Reviews* 37(3):462–476 DOI 10.1111/1574-6976.12011.
Espoz C, Lindberg DR, Castilla JC, Brian Simison W. 2004. Los patelogastrópodos intermareales de Chile y Perú. Revista Chilena De Historia Natural 77:257–283 DOI 10.4067/S0716-078X2004000200006.

Evans RG. 1947. The intertidal ecology of selected localities in the Plymouth neighbourhood. Journal of the Marine Biological Association of the United Kingdom 27:173–218 DOI 10.1017/S0025315400014168.

Fernández M, Jaramillo E, Marquet PA, Moreno CA, Navarrete SA, Ojeda FP, Valdovinos CR, Vasquez JA. 2000. Diversity, dynamics and biogeography of Chilean benthic nearshore ecosystems: an overview and guidelines for conservation.

Finke G, Navarrete S, Bozinovic F. 2007. Tidal regimes of temperate coasts and their influences on aerial exposure for intertidal organisms. Marine Ecology Progress Series 343:57–62 DOI 10.3354/meps06918.

Flores G, Cienfuegos R, Navarrete SA. 2019. Beyond tides: surge-dominated submersion regimes on rocky shores of central Chile. Marine Biology 166:92 DOI 10.1007/s00227-019-3539-8.

Fuhrman JA, Comeau DE, Hagstrom A, Chan AM. 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Applied and Environmental Microbiology 54:1426–1429 DOI 10.1128/aem.54.6.1426-1429.1988.

Fukunaga Y, Kurahashi M, Yanagi K, Yokota A, Harayama S. 2008. Acanthopleuribacter pedis gen. nov. sp. nov. a marine bacterium isolated from a chiton, and description of Acanthopleuribacteraceae fam. nov. Acanthopleuribacterales ord. nov. Holophagaceae fam. nov. Holophagales ord. nov. and Holophagae classis nov. in the phylum Acidobacteria. International Journal of Systematic and Evolutionary Microbiology 58:2597–2601 DOI 10.1099/ijs.0.65589-0.

Garrity G, Bell J, Lilburn T. 2005a. Family I, Rhodobacteraceae fam. nov.. Bergey’s Manual of Systematic Bacteriology 2:161.

Garrity GM, Bell JA, Lilburn T. 2005b. Oceanospirillales ord. nov. In: Bergey’s manual of systematic bacteriology. Boston: Springer, 270–323.

Guo F, Huang Z, Huang M, Zhao J, Ke C. 2009. Effects of small abalone, Haliotis diversicolor, pedal mucus on bacterial growth, attachment, biofilm formation and community structure. Aquaculture 293:35–41 DOI 10.1016/j.aquaculture.2009.03.033.

Gupta RS, Mahmood S, Adeolu M. 2013. A phylogenomic and molecular signature based approach for characterization of the phylum Spirochaetes and its major clades: proposal for a taxonomic revision of the phylum. Frontiers in Microbiology 4:217 DOI 10.3389/fmicb.2013.00217.

Hamady M, Knight R. 2009. Microbial community profiling for human microbiome projects: tools, techniques, and challenges. Genome Research 19:1141–1152 DOI 10.1101/gr.085464.108.

Harley CDG, Helmuth BST. 2003. Local- and regional-scale effects of wave exposure, thermal stress, and absolute versus effective shore level on patterns of intertidal zonation. Limnology and Oceanography 48:1498–1508 DOI 10.4319/lo.2003.48.4.1498.

Hawkins S, Moore P, Burrows M, Poloczanska E, Mieszkowska N, Herbert R, Jenkins S, Thompson R, Genner M, Southward A. 2008. Complex interactions in a rapidly
changing world: responses of rocky shore communities to recent climate change. *Climate Research* 37:123–133 DOI 10.3354/cr00768.

Hedlund BP. 2015. Rubritaleaceae fam. nov. In: Krieg NR, Staley JT, Brown DR, Hedlund BP, Paster BJ, Ward NL, Ludwig W, Whitman WB, eds. *Bergey’s manual of systematic bacteriology, 2nd ed., vol. 4 (The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Firmicutes, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes).* New York: Springer, 812.

Herndl GJ, Peduzzi P. 1989. Potential microbial utilization rates of sublittoral gastropod mucus trails. *Limnology and Oceanography* 34:780–784 DOI 10.4319/lo.1989.34.4.0780.

Hidalgo FJ, Firstater FN, Fanjul E, Bazzettica MC, Lomovasky BJ, Tarazona J, Iribarne OO. 2008. Grazing effects of the periwinkle Echinolittorina peruviana at a central Peruvian high rocky intertidal. *Helgoland Marine Research* 62:73–83 DOI 10.1007/s10152-007-0086-3.

Hill AS, Hawkins SJ. 1991. Seasonal and spatial variation of epilithic micro algal distribution and abundance and its ingestion by *Patella Vulgata* on a moderately exposed rocky shore. *Journal of the Marine Biological Association of the United Kingdom* 71:403–423 DOI 10.1017/S0025315400051675.

Huovinen P, Gómez I. 2011. Spectral attenuation of solar radiation in Patagonian fjord and coastal waters and implications for algal photobiology. *Continental Shelf Research* 31:254–259 DOI 10.1016/j.csr.2010.09.004.

Iguchi SM, Aikawa T, Matsumoto JJ. 1982. Antibacterial activity of snail mucus mucin. *Comparative Biochemistry and Physiology Part A: Physiology* 72:571–574 DOI 10.1016/0300-9629(82)90123-2.

Ivanova EP, Sébastien F, Richard C. 2004. Phylogenetic relationships among marine Alteromonas-like proteobacteria: emended description of the family Alteromonadaceae and proposal of Pseudoalteromonadaceae fam. nov. Colwelliaceae fam. nov. Shewanellaceae fam. nov. Moritellaceae fam. nov. Ferrimonadaceae fam. nov. Idiomarinaceae fam. nov. and Psychromonadaceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology* 54:1773–1788 DOI 10.1099/ijs.0.02997-0.

Jensen S, Duperron S, Birkeland N-K, Hovland M. 2010. Intracellular Oceanospirillales bacteria inhabit gills of Acesta bivalves: acesa excavata bivalve bacterium. *FEMS Microbiology Ecology* 74:523–533 DOI 10.1111/j.1574-6941.2010.00981.x.

Kéfi S, Berlow EL, Wieters EA, Joppa LN, Wood SA, Brose U, Navarrete SA. 2015. Network structure beyond food webs: mapping non-trophic and trophic interactions on Chilean rocky shores. *Ecology* 96:291–303 DOI 10.1890/13-1424.1.

Kéfi S, Berlow EL, Wieters EA, Navarrete SA, Petchey OL, Wood SA, Boit A, Joppa LN, Lafferty KD, Williams RJ, Martinez ND, Menge BA, Blanchette CA, Iles AC, Brose U. 2012. More than a meal...integrating non-feeding interactions into food webs. *Ecology Letters* 15:291–300 DOI 10.1111/j.1461-0248.2011.01732.x.

Keller MD, Selvin RC, Claus W, Guillard RR. 1987. Media for the culture of oceanic ultraphytoplankton 1.2. *Journal of Phycology* 23:633–638.
Kellogg CA, Goldsmith DB, Gray MA. 2017. Biogeographic comparison of lophelia-associated bacterial communities in the Western Atlantic reveals conserved core microbiome. *Frontiers in Microbiology* 8:796 DOI 10.3389/fmicb.2017.00796.

Krieg N, Staley J, Brown D, Hedlund B, Paster B, Ward N, Ludwig W, Whitman III W. 2012. In: Krieg N, Staley JT, Brown DR, Hedlund BP, Paster BJ, Ward NL, Ludwig W, Whitman WB, eds. *Bergery’s manual of systematic bacteriology*. 2nd edn. vol. 4. New York: Springer, 358.

Lafferty KD, Allesina S, Arim M, Briggs CJ, De Leo G, Dobson AP, Dunne JA, Johnson PTJ, Kuris AM, Marcogliese DJ, Martinez ND, Memmott J, Marquet PA, McLaughlin JP, Mordecai EA, Pascual M, Poulin R, Thielges DW. 2008. Parasites in food webs: the ultimate missing links: Parasites in food webs. *Ecology Letters* 11:533–546 DOI 10.1111/j.1461-0248.2008.01174.x.

Lee M. 2019. Happy belly bioinformatics: an open-source resource dedicated to helping biologists utilize bioinformatics. *Journal of Open Source Education* 2:53 DOI 10.21105/jose.00053.

Lema KA, Willis BL, Bourne DG. 2014. Amplicon pyrosequencing reveals spatial and temporal consistency in diazotroph assemblages of the *A. millepora* microbiome: Diazotroph communities associated with the coral *Acropora millepora*. *Environmental Microbiology* 16:3345–3359 DOI 10.1111/1462-2920.12366.

Lewis JR. 1964. *The ecology of rocky shores*. London: English Universities Press.

Lim SJ, Davis BG, Gill DE, Walton J, Nachman E, Engel AS, Anderson LC, Campbell BJ. 2019. Taxonomic and functional heterogeneity of the gill microbiome in a symbiotic coastal mangrove lucinid species. *The ISME Journal* 13:902–920 DOI 10.1038/s41396-018-0318-3.

Liu J, Wang Y, Liu Y, Zhang X-H. 2016. *Ahrensia marina* sp. nov. a dimethylsulfoniopropionate-cleaving bacterium isolated from seawater, and emended descriptions of the genus Ahrensia and Ahrensia kielensis. *International Journal of Systematic and Evolutionary Microbiology* 66:874–880 DOI 10.1099/ijsem.0.000805.

Logares R, Lindström ES, Langenheder S, Logue JB, Paterson H, Laybourn-Parry J, Rengefors K, Tranvik L, Bertilsson S. 2013. Biogeography of bacterial communities exposed to progressive long-term environmental change. *The ISME Journal* 7:937–948 DOI 10.1038/ismej.2012.168.

Martiny AC, Treseder K, Pusch G. 2013. Phylogenetic conservatism of functional traits in microorganisms. *The ISME Journal* 7:830–838 DOI 10.1038/ismej.2012.160.

Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry* 339:69–72 DOI 10.1016/j.ab.2004.12.001.

McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 2013. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of
the National Academy of Sciences of the United States of America 110:3229–3236 DOI 10.1073/pnas.1218525110.

McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE 8(4):e61217 DOI 10.1371/journal.pone.0061217.

Mizutani Y, Mori T, Miyazaki T, Fukuzaki S, Tanaka R. 2020. Microbial community analysis in the gills of abalones suggested possible dominance of epsilonproteobacterium in Haliotis gigantea. PeerJ 8:e9326 DOI 10.7717/peerj.9326.

Morris CE, Monier J-M, Jacques M-A. 1998. A technique to quantify the population size and composition of the biofilm component in communities of bacteria in the phyllosphere. Applied and Environmental Microbiology 64:4789–4795 DOI 10.1128/AEM.64.12.4789-4795.1998.

Muñoz R, Rosselló-Móraa R, Amann R. 2016. Revised phylogeny of Bacteroidetes and proposal of sixteen new taxa and two new combinations including Rhodothermaeota phyl. nov. Systematic and Applied Microbiology 39:281–296 DOI 10.1016/j.syapm.2016.04.004.

Navarrete SA, Gelcich S, Castilla JC. 2010. Long-term monitoring of coastal ecosystems at Las Cruces, Chile: defining baselines to develop ecological literacy in a world of change. Revista Chilena De Historia Natural 83:143–157 DOI 10.4067/S0716-078X2010000100008.

Navarrete S, Parragué M, Osiadacz N, Rojas F, Bonicelli J, Fernandez M, Arboleda-Baena C, Baldanzi S. 2020. Susceptibility of different materials and antifouling coating to macrofouling organisms in a high wave-energy environment. Journal of Ocean Technology 15:72–91.

Navarrete SA, Parragué M, Osiadacz N, Rojas F, Bonicelli J, Fernández M, Arboleda-Baena C, Perez-Matus A, Finke R. 2019. Abundance, composition and succession of sessile subtidal assemblages in high wave-energy environments of Central Chile: Temporal and depth variation. Journal of Experimental Marine Biology and Ecology 512:51–62 DOI 10.1016/j.jembe.2018.12.006.

Nedashkovskaya OI, Ludwig W. 2015. Cyclobacteriaceae fam. nov. In: Krieg NR, Staley JT, Brown DR, Hedlund BP, Paster BJ, Ward NL, Ludwig W, Whitman WB, eds. Bergey’s Manual of Systematic Bacteriology, 2nd ed., vol. 4 (The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes). New York: Springer-Verlag, 423.

Neu AT, Allen EE, Roy K. 2019. Diversity and composition of intertidal gastropod microbiomes across a major marine biogeographic boundary. Environmental Microbiology Reports 11:434–447 DOI 10.1111/1758-2229.12743.

Nicotri ME. 1977. Grazing effects of four marine intertidal herbivores on the microflora. Ecology 58:1020–1032 DOI 10.2307/1936922.

Oksanen J, Blanchet F, Kindt R, Legendre P, Minchin P, O’Harra R, Simpson G, Solymos P, Stevens M, Wagner H. 2015. Vegan: community ecology package. R
package vegan, vers. 2.2-1. Available at https://cran.r-project.org/web/packages/vegan/index.html.

Otaíza R, Santelices B. 1985. Vertical distribution of chitons (Mollusca: Polyplacophora) in the rocky intertidal zone of central Chile. *Journal of Experimental Marine Biology and Ecology* 86:229–240 DOI 10.1016/0022-0981(85)90105-4.

Pantos O, Bongaerts P, Dennis PG, Tyson GW, Hoegh-Guldberg O. 2015. Habitat-specific environmental conditions primarily control the microbiomes of the coral *Seriatopora hystrix*. *The ISME Journal* 9:1916–1927 DOI 10.1038/ismej.2015.3.

Parada AE, Needham DM, Fuhrman JA. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples: primers for marine microbiome studies. *Environmental Microbiology* 18:1340–1414 DOI 10.1111/1462-2920.13023.

Pfister CA, Meyer F, Antonopoulos DA. 2010. Metagenomic profiling of a microbial assemblage associated with the California mussel: a node in networks of carbon and nitrogen cycling. *PLOS ONE* 5(5):e10518 DOI 10.1371/journal.pone.0010518.

Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ. 2011. Removing noise from pyrosequenced amplicons. *BMC Bioinformatics* 12:38 DOI 10.1186/1471-2105-12-38.

R Core Team. 2013. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at https://www.r-project.org.

Racine JS. 2012. RStudio: a platform-independent IDE for R and sweave. *Journal of Applied Econometrics* 27(1):167–172 DOI 10.1002/jae.1278.

Rillig MC, Antonovics J, Caruso T, Lehmann A, Powell JR, Veresoglou SD, Verbruggen E. 2015. Interchange of entire communities: microbial community coalescence. *Trends in Ecology & Evolution* 30:470–476 DOI 10.1016/j.tree.2015.06.004.

Ribes M, Calvo E, Movilla J, Logares R, Coma R, Pelejero C. 2016. Restructuring of the sponge microbiome favors tolerance to ocean acidification: acidification and Mediterranean sponges. *Environmental Microbiology Reports* 8:536–544 DOI 10.1111/1758-2229.12430.

Rigler RJ. 1966. Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by acridine orange. *Acta Physiologica Scandinavica* 67:1–122 DOI 10.1111/j.1748-1716.1966.tb03280.x.

Rillig MC, Antonovics J, Caruso T, Lehmann A, Powell JR, Veresoglou SD, Verbruggen E. 2015. Interchange of entire communities: microbial community coalescence. *Trends in Ecology & Evolution* 30:470–476 DOI 10.1016/j.tree.2015.06.004.

Rivadeneira M, Fernández M, Navarrete S. 2002. Latitudinal trends of species diversity in rocky intertidal herbivore assemblages: spatial scale and the relationship between local and regional species richness. *Marine Ecology Progress Series* 245:123–131 DOI 10.3354/meps245213.

Roberts DW, Roberts MDW. 2016. Package ‘labdsv’. *Ordination and Multivariate Analysis in R*. 775:1–68.

Rosenberg E, Zilber-Rosenberg I. 2016. Microbes drive evolution of animals and plants: the hologenome concept. *mBio* 7(2):e01395 DOI 10.1128/mBio.01395-15.

Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME Journal* 4:1340–1351 DOI 10.1038/ismej.2010.58.
Salta M, Wharton JA, Stoodley P, Dennington SP, Goodes LR, Werwinski S, Mart U, Wood RJK, Stokes KR. 2010. Designing biomimetic antifouling surfaces. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 368:4729–4754 DOI 10.1098/rsta.2010.0195.

Santelices B. 1990. Patterns of organizations of intertidal and shallow subtidal vegetation in wave exposed habitats of central Chile. Hydrobiologia 192:35–57 DOI 10.1007/BF00006226.

Santelices B, Bobadilla M. 1996. Gastropod pedal mucus retains seaweed propagules. Journal of Experimental Marine Biology and Ecology 197:251–261 DOI 10.1016/0022-0981(95)00151-4.

Santelices B, Vásquez J, Meneses I. 1986. Patrones de distribución y dietas de un gremio de moluscos herbívoros en hábitats intermareales expuestos de Chile central. In: Santelices B, ed. Monografías Biológicas Simposio Internacional Usos y funciones de las algas marinas bentónicas. Santiago: Pontificia Universidad Católica de Chile, 147–171.

Shade A, Handelsman J. 2012. Beyond the Venn diagram: the hunt for a core microbiome: the hunt for a core microbiome. Environmental Microbiology 14:4–12 DOI 10.1111/j.1462-2920.2011.02585.x.

Shashoua VE, Kwart H. 1959. The structure and constitution of mucus substances, II. The chemical constitution of busycon mucus. Journal of the American Chemical Society 81:2899–2905 DOI 10.1021/ja01520a069.

Stephenson TA, Stephenson A. 1961. Life between tide-marks in North America, IVB. Vancouver Island, II. The Journal of Ecology 49:227 DOI 10.2307/2257258.

Szathmary P, Helmhuth B, Wethey D. 2009. Climate change in the rocky intertidal zone: predicting and measuring the body temperature of a keystone predator. Marine Ecology Progress Series 374:43–56 DOI 10.3354/meps07682.

Thompson RC, Roberts MF, Norton TA, Hawkins SJ. 2000. Feast or famine for intertidal grazing molluscs: a mis-match between seasonal variations in grazing intensity and the abundance of microbial resources. Hydrobiologia 440:357–367 DOI 10.1023/A:1004116505004.

Tremaroli V, Bäckhed F. 2012. Functional interactions between the gut microbiota and host metabolism. Nature 489:242–249 DOI 10.1038/nature11552.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JJ. 2007. The human microbiome project. Nature 449:804–810 DOI 10.1038/nature06244.

Underwood AJ, Denley EJ, Moran MJ. 1983. Experimental analyses of the structure and dynamics of mid-shore rocky intertidal communities in New South Wales. Oecologia 56:202–219 DOI 10.1007/BF00379692.

Vasconcelos V. 2020. Distinct temporal succession of bacterial communities in early marine biofilms in a Portuguese Atlantic Port. Frontiers in Microbiology 11:17 DOI 10.3389/fmicb.2020.00017.

Villasante A, Catalán N, Rojas R, Lohrmann KB, Romero J. 2020. Microbiota of the digestive gland of red abalone (haliotis rufescens) is affected by withering syndrome. Microorganisms 8:1411 DOI 10.3390/microorganisms8091411.
Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. 2012. The second skin: ecological role of epibiotic biofilms on marine organisms. *Frontiers in Microbiology* 3:292 DOI 10.3389/fmicb.2012.00292.

Walters AD, Arp A, Cerbie GM, Trujillo DA, Kiss AJ, Berg DJ. 2022. Phylogenetic relationship and habitat both impact the gut microbiome in two microendemic gastropods. *Journal of Molluscan Studies* 88:eyac002 DOI 10.1093/mollus/eyac002.

Wethey DS. 1983. Geographic limits and local zonation: the barnacles Semibalanus (Balanus) and Chthamalus in New England. *The Biological Bulletin* 165:330–341 DOI 10.2307/1541373.

Williams G, Morritt D. 1995. Habitat partitioning and thermal tolerance in a tropical limpet, Cellana grata. *Marine Ecology Progress Series* 124:89–103 DOI 10.3354/meps124089.

Zbinden M, Marqué L, Gaudron SM, Ravaux J, Léger N, Duperron S. 2015. Epsilonproteobacteria as gill epibionts of the hydrothermal vent gastropod Cytheremia naticoides (North East-Pacific Rise). *Marine Biology* 162:435–448 DOI 10.1007/s00227-014-2591-7.