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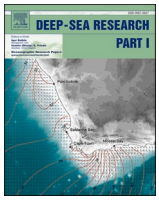

Faunal colonists, including mussel settlers, respond to microbial biofilms at deep-sea hydrothermal vents

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ABSTRACT

Colonization processes at dynamic deep-sea hydrothermal vent ecosystems ultimately determine ecosystem structure, function, resilience, and recovery. Microbial biofilms form rapidly on surfaces near hydrothermal vents and are continuously exposed to the highly variable abiotic environment. Thus, biofilm microbes may provide a temporally integrated signal that can indicate whether the habitat is suitable for faunal colonists. This study explored the role of microbial biofilms in controlling faunal colonization through *in-situ* colonization experiments at Tica Vent in the 9°50' N region of the East Pacific Rise (EPR). Short-term experiments (~2 weeks) were conducted by deploying colonization surfaces ("sandwiches") either with an established biofilm (developed for >1 year) or a fresh biofilm (developed throughout experiment) in zones characterized by different faunal assemblages. Differences in associated larval settlers, faunal immigrants, and microbial communities according to biofilm age across multiple biogenic zones were investigated. Faunal and microbial community compositions significantly differed according to whether the sandwiches had established or fresh biofilms as well as the biogenic zone they were deployed in. Several faunal colonists, including settlers such as the foundational chemosymbiotic mussel *Bathymodiolus thermophilus* and the nectochaete *Archinome* sp., were found associated more with established biofilms than fresh biofilms. Microbial biofilm communities were dominated by putative chemoautotrophic members of the Campylobacterota phylum and Gammaproteobacteria class and several microbial taxa were found to covary with faunal colonists. Overall, these findings show that microbial community composition plays a role in larval settlement and animal migration in hydrothermal vent systems and the detection of microbial and faunal interactions provides a starting point for identifying key microbial characteristics influencing colonization processes at hydrothermal vents.

1. Introduction

Deep-sea hydrothermal vents are productive island-like habitats that can be subject to frequent natural disturbances and are potential targets for deep-sea mining operations (Van Dover et al., 2018). Due to their inherent spatial patchiness and extreme variability on small temporal and spatial scales, the colonization of organisms at hydrothermal vents is a key process affecting ecosystem function, resilience, and recovery (Mullineaux et al., 2018, 2020; Dykman et al., 2021). Physico-chemical factors have been suggested to be the main drivers of microbial and faunal community structure and distribution near hydrothermal vents (Shank et al., 1998; Desbruyères et al., 2001; Luther et al., 2001) due to the extreme and highly variable abiotic conditions hydrothermal vent organisms experience. Indeed, previous work has shown that both faunal and biofilm microbial community colonization and succession patterns are shaped by abiotic conditions such as temperature, pH, and H_2S and O_2 concentrations (Shank et al., 1998; Marcus et al., 2009; Sen et al., 2014; O'Brien et al., 2015; Sciutteri et al., 2022). After seafloor eruptions, previous work has demonstrated that hydrothermal vent systems are initially colonized by microbial communities and faunal

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colonization follows (Shank et al., 1998; Alain et al., 2004). Microbial biofilms are rapidly established, within days, on freshly exposed surfaces near diffuse hydrothermal flow (Guezennec et al., 1998; Alain et al., 2004; Gulmann et al., 2015). The three-dimensional biofilm matrix is comprised of microbial cells embedded in self-produced extracellular polymeric substances (EPSs) containing polysaccharides, proteins, and nucleic acids (Sutherland, 2001; Costa et al., 2018), thus creating a protective environment in which microbes can maintain access to nutrients and energy sources within hydrothermal fluids (Sievert and Vetriani, 2012; Zykwinska et al., 2019). Biofilm characteristics such as microbial biomass concentrations, microbial community composition, and physical and chemical alteration of surfaces have been suggested to play key roles in "conditioning" the habitat for faunal colonization (Adams et al., 2012; Sievert and Vetriani, 2012; O'Brien et al., 2015).

The ability of vent endemic invertebrate larvae to find and successfully colonize 'the right' habitat is critical for the survival of vent populations due to the steep gradients and temporal variability in physical and chemical conditions and food availability (Adams et al., 2012). The animals colonizing these habitats must select a location that accommodates their physiological tolerances and nutritional requirements. In other marine systems, microbial biofilms have been shown to mediate settlement in a variety of invertebrate larvae, including mussels and polychaetes (reviewed in Hadfield, 2011; Dobretsov and Rittschof, 2020), thus indicating that biofilms provide cues that larvae can use to assess the suitability of the settlement site. Larvae have been shown to initiate settlement and metamorphosis based on the presence and abundance of specific microbial taxa (Huang and Hadfield, 2003) and the chemical compounds produced by microbes (Tait and Havenhand, 2013; Sneed et al., 2014; Freckelton et al., 2022). The specific cues appear to vary by invertebrate species and complex, natural biofilms often stimulate larval settlement better than monospecific biofilms (Huang and Hadfield, 2003; Hadfield, 2011). Biofilms may also increase the ability of larvae to firmly attach to surfaces (Zardus et al., 2008; Hadfield, 2011). Additionally, many hydrothermal vent invertebrates rely on chemosynthetic endosymbionts for nutrition and acquire their symbionts from the environment (Vrijenhoek, 2010). Therefore, the presence of free-living symbiont populations in the water column or in microbial biofilms must precede colonization of dependent invertebrate populations. For animals that do not purely rely on symbiosis for their nutritional requirements, biofilms provide a consistent source of concentrated microbial biomass and organic carbon that is available for consumption by higher trophic levels including various vent invertebrates (Govenar, 2012). This food source may also impact faunal colonization via attraction of both faunal settlers and immigrants from surrounding areas.

Despite the strong abiotic influence, previous work has demonstrated that biotic factors also influence faunal colonization and succession patterns near hydrothermal vents (Mullineaux et al., 2000, 2003, 2012; Micheli et al., 2002). For example, the presence of a pioneer species was shown to facilitate settlement of other species (Mullineaux et al., 2000) and the experimental exclusion of predators near hydrothermal vents altered the benthic community composition across a gradient of vent fluid flux environments (Micheli et al., 2002). Additionally, the succession of faunal communities after two seafloor eruptions differed in ways that could not be explained by variations in abiotic factors alone suggesting that larval dispersal and species interactions played important roles in the recovery of ecosystem structure and function after a disturbance (Mullineaux et al., 2012). Although the importance of biotic interactions in structuring hydrothermal vent communities has become clear, very little is known about how free-living microorganisms, including microbial biofilms, interact with fauna to shape hydrothermal-vent communities.

The East Pacific Rise (EPR) is one of the best studied hydrothermal vent systems with relatively long-term time series observations and documentation of ecosystem changes after multiple seafloor eruptions (e.g., Shank et al., 1998; Fornari et al., 2012; Gollner et al., 2020; Mullineaux et al., 2020; Dykman et al., 2021). Even after 11 years from the most recent eruption, ecological communities were still shifting and differed from the pre-eruption state suggesting that an equilibrium community had not been reached and colonization processes were still influencing community assembly (Mullineaux et al., 2020). Currently at the EPR, more than 12 years after the last eruption, foundational vent invertebrates including *Alvinella pompejana*, *Riftia pachyptila*, and *Bathymodiolus thermophilus* occupy distinct niches along a gradient of hydrothermal flow (Micheli et al., 2002; Le Bris et al., 2005; Le Bris and Gaill, 2007; Mullineaux, 2013). It is well established that faunal community composition varies across these biogenic zones (Galkin and Goroslavskaya, 2008; Gollner et al., 2010, 2015; Mullineaux et al., 2012) and that microbial biofilm communities are altered by changes in hydrothermal flow (O'Brien et al., 2015), but it is unclear whether microbial biofilms influence these faunal distribution and colonization patterns within and across zones with differing abiotic conditions.

Due to large variations in the physico-chemical conditions on small spatial and temporal scales, microbial communities are expected to provide an integrated and, therefore, more reliable signal of the range of conditions experienced at a given location. This integrated picture may provide animals with essential information related to their physiological and nutritional needs. Thus, to elucidate the roles of microbial biofilms in faunal colonization at a productive but disturbance-prone deep-sea ecosystem, this work utilized deep-sea submersible and remotely operated vehicle technology to conduct *in-situ* colonization experiments in well-established communities at the EPR. Experiments were designed to provide potential colonists with both established and fresh biofilmed surfaces across a gradient of physico-chemical conditions to explore whether short-term faunal colonization patterns, especially larval settlement, are influenced by microbial biofilms.

2. Methods

2.1. Study site and experiment details

A short-term in-situ colonization experiment was conducted at Tica Vent (9°50.3987 N, 104°17.4970 W) in the 9°50' N region of the EPR. Colonization surfaces, comprised of 6 stacked polycarbonate plates separated by spacers and termed "sandwiches" (described by Mullineaux et al., 2010; Dykman et al., 2021, Fig. 1), were deployed by the deep submergence vehicles HOV *Alvin* and ROV *Jason* over two cruises; one aboard the R/V *Atlantis* in December 2019 and the other on the R/V *Roger Revelle* in March–April 2021. During the first deployment in December 2019, 12 sandwiches were covered in 200 μm nylon mesh bags termed "purses" to prevent animal colonization while allowing for microbial biofilm development (Fig. 1). The purses were custom designed with handles and completely enclosed around the sandwich with hook and loop fasteners so that they could be removed *in-situ* by vehicle manipulators (L. Lavigne, Anacortes, WA). Four replicate "pursed sandwiches" were placed within each of three biogenic zones (*Alvinella*-dominated, *Riftia*-dominated, and mussel-dominated) that span a gradient of temperature and chemical conditions (Micheli et al., 2002; Le Bris et al., 2005; Mullineaux, 2013).

The colonization experiment was initiated when the ROV *Jason* revisited the pursed sandwiches approximately 15 months later. Several pursed sandwiches (3 from the Alvinellid zone and 2 from the Riftia zone) were lost due to overgrowth by animal communities, resulting in the discovery of 7 of the initial 12 pursed sandwiches. For the 7 pursed sandwiches that were discovered, purses were removed, and the established biofilm sandwiches were placed back in the location they had been found. Fresh sandwiches (no previously developed biofilm) were deployed next to each of the 7 established biofilm sandwiches to act as paired controls. The sandwiches were left on the seafloor for approximately 15 days to allow for animal colonization. After which, they were recovered into separate sealed collection compartments on the ROV *Jason*. Although most sandwiches were brought to the surface before

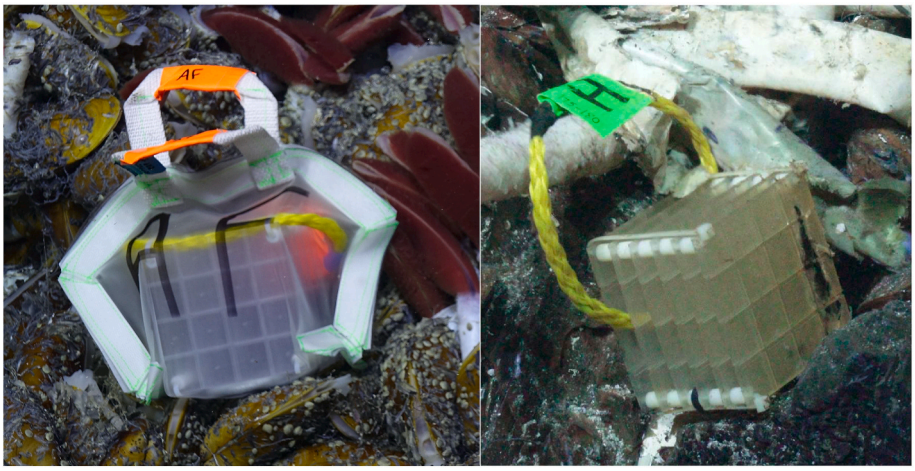

Fig. 1. Images of colonization sandwiches deployed either with (left) or without (right) a 200 μm mesh "purse".

preservation, 1 established biofilm sandwich from each zone (including the only established biofilm sandwich in the Alvinellid zone) was recovered into individual cylinders specifically designed for immediate preservation of samples *in-situ* (Analytical Instrument Systems, Ringoes, NJ) that were filled with an RNA stabilization solution (25 mM sodium citrate, 10 mM EDTA, 70 g ammonium sulfate per 100 mL solution, adjusted to pH 7.0 with sulfuric acid); preservation in the RNA stabilization buffer makes these samples available for other work not reported here. A temperature probe held at the base of each sandwich for approximately 1–2 min was used to record the temperature maximum at deployments and recoveries of all sandwiches.

2.2. Shipboard preservation and processing of sandwiches

Upon vehicle recovery, containers holding sandwiches were transported to a cold room (4 °C). Attached weights and polypropylene handles were removed and the zipties holding each sandwich together were cut to remove the top sandwich plate. Any animals visible on the top plate were gently removed and placed into a separate container filled with RNA preservative. The top plate was then placed into a plastic bag with enough RNA preservative to cover the entire plate and frozen at −80 °C until further processing for 16S rRNA gene sequencing. The remaining parts of the sandwich were placed into separate plastic bags, submerged in RNA preservative, and stored at 4 °C until further processing for animal colonization. To collect any fauna that had fallen off the sandwiches, seawater or RNA preservative in the sandwich collection compartments was poured and rinsed over a 63 μm mesh sieve, then stored in RNA preservative at 4 °C until further processing.

2.3. Characterizing animal colonist communities

At Western Washington University's Shannon Point Marine Center (SPMC) faunal colonists from each sandwich were observed and counted under a stereo microscope (Olympus) at 4x magnification. Both sides of each sandwich plate were carefully inspected under a microscope and attached organisms were removed and counted. Additionally, any organisms in the RNA stabilization solution in the containers holding the sandwich plates and sieved sandwich compartment contents were sorted and counted. Individual colonists were grouped into morphotypes based on visual features while any damaged organisms that could not be assigned to a morphotype group were grouped into a broad unknown taxonomic group (e.g., unknown polychaete, unknown gastropod, etc.). Representative organisms from morphotype groups were imaged on either a stereo microscope (Leica; magnification range 0.8–10x) or a compound microscope (Leica; 20x magnification) for possible further taxonomic identification and to create a morphotype guide (Supplementary Guide S1).

A count table of colonist morphotypes (Supplementary Data Table S1) was used to explore trends in faunal community composition associated with sandwiches across biogenic zones and biofilm age. A combination of model-based and distance-based multivariate analyses were conducted in R (v 4.1.1) to explore colonist community composition. Model-based analyses have recently become more prevalent in multivariate ecology and have been suggested to have high-power and flexibility while accounting for mean-variance relationships in count data (Wang et al., 2012; Warton et al., 2012). Multivariate generalized linear models (GLMs) were constructed with the R function manyglm (mvabund R package; Wang et al., 2012) using combinations of biogenic zone, biofilm age, and their interaction as predictor variables and morphotype counts as response variables. Morphotype abundance data was assumed to follow a negative binomial distribution (family = "negative.binomial"). Model selection was based on minimizing the summed AIC and assessing whether additional variables significantly improved the model by explaining additional variance (ANOVA $p < 0.05$). Significance of predictor variables were assessed using the anova.manyglm function, which provides a multivariate test for the overall community composition and univariate tests for each faunal morphotype. Multivariate test statistics were calculated using the Score statistic (test = "score") while accounting for correlations between morphotypes (cor.type = "shrink"). P-values were calculated with the PIT-trap bootstrap resampling method. Univariate tests were adjusted for multiple testing using p. uni = "adjusted".

Since distance-based approaches to analyzing high-dimensional ecological data have been well established and have been shown to reliably detect differences in community structure (Roberts, 2017), these methods were also used and compared to model-based approaches. A Bray-Curtis dissimilarity matrix was created from the square root transformed morphotype count table and was used for visualization of faunal community composition differences via principal coordinates analysis (PCoA) and statistical analysis of differences in faunal community composition based on biogenic zone and biofilm age via permutational multivariate analysis of variance (PERMANOVA) (vegan and pairwise.adonis R packages; Oksanen et al., 2013; Martinez Arbizu, 2020). Bray-Curtis dissimilarity measures were used because of the asymmetrical properties and the ability to handle ecological count data well. Although temperature measurements were only taken briefly under each sandwich at the experiment start and recovery, temperatures are often used as a proxy for hydrothermal fluid flow and should correlate with biogenic zones, so temperature measurements (Supplementary Data Table S1) were fit to the first two axes of the PCoA plot (envfit function) to assess whether temperature appears to be an important factor influencing community composition. Further analysis

of individual morphotype abundance differences between pairs of established and fresh biofilm sandwiches were analyzed with paired t-tests or Wilcoxon signed rank tests (if counts were not approximately normally distributed based on the Shapiro-Wilk test of normality).

2.4. Biofilm DNA extraction and 16S rRNA gene sequencing

To remove biofilms from the sandwich plates in preparation for DNA extraction, a sterile razor blade was used to aseptically loosen the biofilm prior to agitation of the plate while submerged in RNA preservative. Following agitation, approximately 50 mL of RNA preservative per plate was centrifuged in Corex tubes at 8349 rpm for 1 h at 4 °C. Supernatant was removed and the pellet was washed twice in 1X PBS and stored at −20 °C before proceeding to DNA extraction. For DNA extraction, a modified phenol-chloroform extraction technique was used (Patwardhan et al., 2021). Extracted DNA was purified using a DNA Clean & Concentrator−5 Kit (Zymo Research) following the manufacturer's instructions. Concentrations of purified DNA were determined using the Qubit® dsDNA HS Assay Kit (Life Technologies).

Amplification, library preparation, and sequencing of the V4 region of the 16S rRNA gene was performed by Molecular Research, LP (www.mrdnalab.com, Shallowater, TX). Briefly, extracted genomic DNA was amplified using prokaryotic universal primers (515Y 5′-GTG YCA GCM GCC GCG GTA A-3′ and 806 R 5′-GGA CTA CNV GGG TWT CTA AT-3′) (Apprill et al., 2015; Parada et al., 2016) with the HotStarTaq Plus Master Mix kit (Qiagen, USA) in a 30-cycle polymerase chain reaction (PCR) with thermal cycling conditions as follows: 95 °C for 5 min, 30 cycles of: 95 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min; and a final elongation at 72 °C for 10 min. Following amplification, PCR products were checked in 2% agarose gel and samples were multiplexed using unique dual indices and pooled in equal proportions based on molecular weight and DNA concentrations. Pooled samples were purified with Ampure XP beads. Library sequencing was performed on an Illumina MiSeq (2 × 300 bp).

2.5. Processing and analysis of microbial biofilm sequencing data

Demultiplexed sequencing reads obtained from Molecular Research, LP were processed with the DADA2 pipeline (Callahan et al., 2016) in R (v. 4.3.1). Based on package guidelines, sequences were first evaluated according to their quality and then filtered and trimmed to remove primer sequences and low quality regions (truncLen = c (210,240), maxEE = c (3,3), truncQ = 2, trimLeft = c (19,20)). To overcome the effect of the binned quality scores obtained from the sequencing run, error estimation was determined with an adapted version of the learnErrors function "errorEstimationFunction" which enforces the monotonicity of the error model by changing the arguments of the loess model to have a span equal to 2 and weights equal to the log-transformed total counts of nucleotides (see https://github.com/benjjneb/dada2/issues/1307). Then, paired-end reads were merged and Amplicon Sequence Variants (ASVs) were obtained. Sequences shorter than 252 bp or longer than 255 bp were discarded. Chimeric sequences were removed (minFoldParentOverAbundance = 10, method = "consensus") and taxonomy was assigned using the naïve Bayesian classifier method (Wang et al., 2007) against the Silva Database (r138.1; https://www.arb-silva.de/documentation/release-1381/). Before further analysis, sequences belonging to Eukaryotes, Mitochondria, and Chloroplasts were removed. The resulting dataset contained a total of 2,934,072 reads in 6053 unique ASVs for downstream analyses. Sequencing data was analyzed in R using phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2013) packages.

Visualization of ASV richness in relation to sequencing library size for each sample was used to determine if there was sufficient sequencing depth to recover biofilm communities (Fig. S1). Sequences were converted to relative abundances before analysis of community composition. The effect of biogenic zone and biofilm age on ASV richness and Shannon diversity were explored using analysis of variance (ANOVA) methods after testing for normality of residuals (Shapiro-Wilk test) and homogeneity of variances across groups (Brown-Forsyth test). The effect of biofilm age on ASV richness and Shannon diversity was further explored with paired t-tests (data was approximately normally distributed, Shapiro-Wilk test) or paired Wilcoxon signed rank tests (non-normal data, Shapiro-Wilk test).

Principal coordinates analysis (PCoA) and PERMANOVA (adonis2 and pairwise. adonis2 functions) using Bray-Curtis dissimilarity measures were used to visualize (PCoA) and test for (PERMANOVA) differences in microbial community composition based on biogenic zone and biofilm age. Bray-Curtis dissimilarity measures were chosen because of the weighted asymmetrical properties and interpretable limits that are suitable for sparse relative abundance data. Multivariate modeling approaches were not used for the compositional microbial community data because these data can only be assessed as relative abundances and cannot be modelled as absolute count data with the same approaches used for the faunal count data described above. Sequencing library size was also tested to make sure it was not a significant factor impacting community composition (PERMANOVA, $p > 0.05$). Temperatures measured upon experiment start and recovery (Supplementary Data Table S1) were fit to the first two ordination axes of the PCoA plot (envfit function) to test whether environmental gradients significantly influenced community composition.

To examine broad taxonomic trends in community composition differences due to biofilm age, differences in the top family-level relative abundances between established and fresh biofilm sandwiches were tested using paired t-tests or paired Wilcoxon signed rank tests. For more resolved taxonomic patterns, differential ASV abundance between established and fresh biofilm sandwiches were determined with Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020). The non-normalized ASV table was filtered to remove ASVs that were present in fewer than 3 sandwich samples and never had greater than 0.1% relative abundance in a sample, resulting in differential abundance analysis of 450 ASVs. Biogenic zone was included as a co-variate in the ANCOM-BC log-linear regression model and p-values were corrected for multiple testing with the Benjamin-Hochberg correction. Since published 16S rRNA gene sequences are available for the symbionts within the foundational fauna dominating each of the biogenic zones and environmental populations of these symbionts may be critical to colonization, we used published sequences from a gill endosymbiont of adult *B. thermophilus* (Distel et al., 1988), the trophosome symbiont of *R. pachyptila* (Distel et al., 1988), and an epibiont of *A. pompejana* (Haddad et al., 1995) to search for potential matches to ASVs in this dataset. The relative abundance and prevalence of the ASVs found to match these symbiont sequences were analyzed according to biogenic zone and biofilm age.

2.6. Proportionality analysis of faunal colonists and microbes

Proportionality analysis, a compositionally aware method to measure association, (propr package; Quinn et al., 2017) was used to identify covariance in ratios between faunal colonists and microbial taxa. Both the colonist count table and the microbial ASV table (non-normalized) were filtered to remove low abundance (colonist counts <5, ASV relative abundance <0.5%) and low prevalence taxa (<3 samples) to minimize the risk of spurious correlations and allow for interpretation. The resulting tables including 25 individual colonist morphotypes and 118 microbial ASVs were converted to pseudo-counts by imputation of zero-values (zCompositions package; Palarea-Albaladejo and Martín-Fernández, 2015) and center log-ratio (clr) transformed as in Murdock et al. (2021). Pairwise covariances ($|\rho| > 0.6$) between faunal colonists and microbial ASVs were visualized according to broad taxonomic group patterns with the circlize package (Gu et al., 2014). Additionally, a pairwise correlation test (cor.test, method = "spearman") was conducted between clr transformed pseudo-counts of *B. thermophilus*

post-larvae and an ASV that closely matched a sequenced gill endosymbiont of adult *B. thermophilus* and was not included in the proportionality analysis.

3. Results

3.1. Colonist community composition according to biofilm age and across biogenic zones

At a broad taxonomic level, faunal communities associated with sandwiches were dominated by gastropods, copepods, amphipods, and polychaetes (Fig. 2). For each sandwich, most of the identified colonists were juveniles or adults of benthic fauna or holoplankton while only a small percentage of colonists (0–12%) were classified as potential settlers (bivalve veligers, gastropod veligers, and nectochaetes) (Fig. 2). Counts of colonists across individual sandwiches varied significantly, ranging from 61 identified individuals associated with the established biofilm sandwich in the Alvinellid zone to 937 identified individuals associated with an established biofilm sandwich from the *Riftia* zone. The most abundant morphotypes present across all sandwiches were putatively identified as the limpet *Lepetodrilus elevatus*, the amphipod, *Ventiella sulfuris*, copepods belonging to the Dirivulidae family and Harpacticoida order, and the polychaetes, *Ophryotrocha akessoni* and *Archinome* spp. (Supplementary Guide S1). One of the sandwich pairs from the *Riftia* zone had large numbers of limpets, mostly *Lepetodrilus* sp., that dominated the colonist counts and were more abundant than all combined colonists on every other sandwich.

When considering colonist communities as a whole, statistical differences in community composition were detected through both distance and model-based approaches based on whether the sandwich had an established or fresh biofilm (PERMANOVA $p = 0.018$, GLM ANOVA $p = 0.010$), as well as across biogenic zones (PERMANOVA $p = 0.005$, GLM ANOVA $p = 0.039$) (Fig. 3,Supplementary Tables S1–S2). Significant multiple linear regressions were detected between the first two ordination axes of the PCoA plot (Fig. 3,Supplementary Fig. S2) and the temperature measured at the base of each sandwich at either the start of the experiment ($p = 0.030$) or upon sandwich recovery ($p = 0.001$) suggesting that temperature (a proxy for exposure to hydrothermal fluids, which should also be associated with biogenic zone) is an important factor in controlling faunal colonist community composition (Supplementary Fig. S2). Model selection based on summed AIC revealed that the best performing multivariate GLM included both biogenic zone and biofilm age, but not their interaction (Counts ~ Zone + Biofilm Age) (Supplementary Table S3). When comparing the model containing just biogenic zone (base model) to the model containing both zone and biofilm age, significant differences were detected (ANOVA $p = 0.024$) suggesting that biofilm age significantly improves the model and explains additional variance in the count data (Supplementary Table S3). Pairwise comparisons of community composition across biogenic zones (pairwise PERMANOVAs) identified significant differences between mussel and Riftia zones ($p = 0.007$) and mussel and Alvinellid zones ($p = 0.047$) but not between Alvinellid and *Riftia* zones

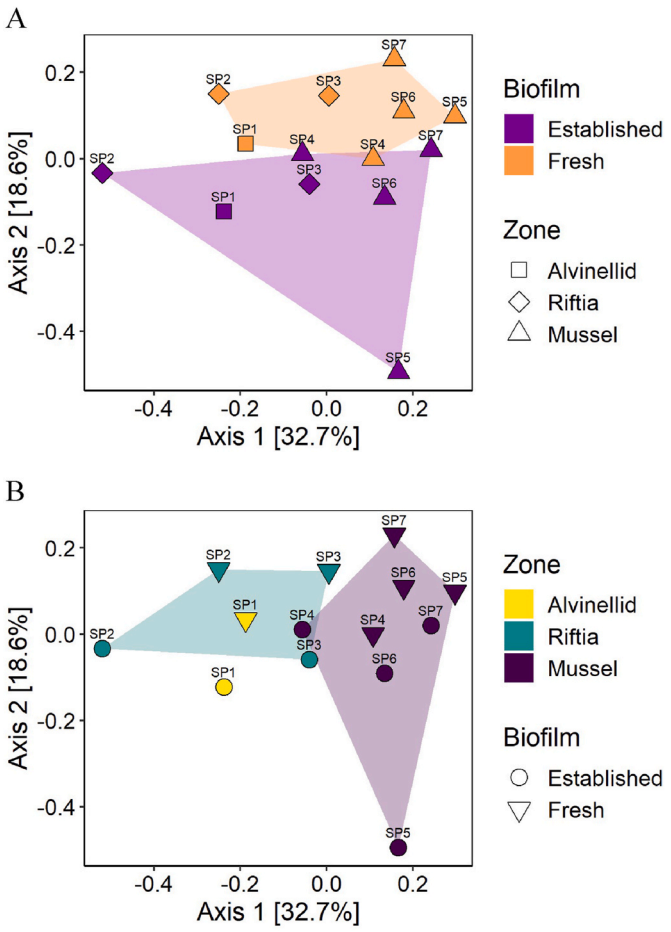

Fig. 3. Principal coordinates analysis (PCoA) of the Bray-Curtis dissimilarity matrix based on square root transformed faunal colonist counts in each sandwich sample. A. Sandwich samples (points) are colored by biofilm age and shaped by biogenic zone. B. Sandwich samples (points) are colored by biogenic zone and shaped by biofilm age.

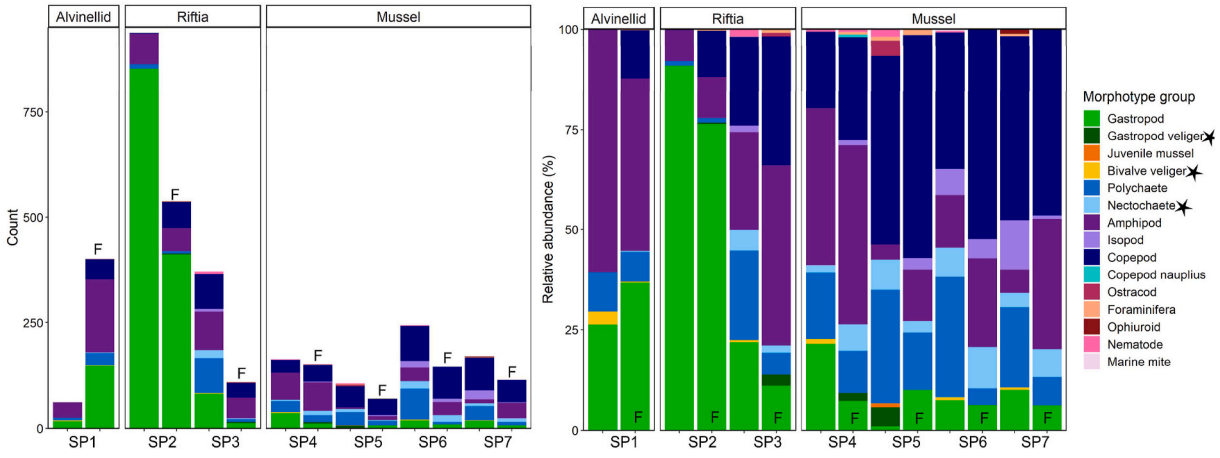

Fig. 2. Faunal colonist counts (left) and relative abundances (right) grouped by broad taxonomic classifications. Each sandwich pair (SP) is plotted together and grouped by biogenic zone with the fresh biofilm pair denoted with an "F". Potential settlers are marked with a star in the legend.

(p = 0.6). Note that due to limited sample size in the Alvinellid zone (only 1 sandwich pair), the statistical power to detect differences compared to the Alvinellid zone is low. Additionally, univariate tests in the optimum multivariate GLM did not identify any morphotypes that significantly varied across biogenic zone (all padj >0.1, Supplementary Table S2) suggesting the model had low power to identify drivers of community change across zone. In the mussel and *Riftia* zones, all fresh sandwich pairs had fewer colonists than their established biofilm pair (ranging from 11 fewer to 399 fewer). Conversely, for the Alvinellid zone, the fresh sandwich had 340 more colonists than the established biofilm pair. When considering only sandwich pairs from the *Riftia* and mussel zones, total counts of juvenile and adult polychaetes were statistically greater on established biofilm sandwiches compared to fresh biofilm sandwiches (paired *t*-test p = 0.038).

Across all sandwich pairs, several individual colonist morphotypes were detected in higher abundances with established compared to fresh biofilm sandwiches (Fig. 4). Statistically significant differences in potential settler abundances between paired established and fresh biofilm sandwiches were detected for a bivalve identified as *Bathymodiolus thermophilus* (paired *t*-test p = 0.015) and a nectochaete identified as *Archinome* sp. (paired *t*-test p = 0.045). Additionally, polychaetes including *Amphisamytha galapagensis* and a morphotype belonging to the Polynoidae family were statistically more abundant in association with established biofilm sandwiches compared to their fresh paired sandwich (paired *t*-test p = 0.024 and p = 0.045, respectively). Univariate tests for each morphotype in the optimum multivariate GLM model also confirmed that *B. thermophilus* post-larvae and *A. galapagensis* polychaetes had significant (or marginally significant) differences in counts according to biofilm age (GLM padj = 0.047 and padj = 0.080, respectively) (Supplementary Table S2) and were likely important drivers for community differences.

3.2. Microbial community composition according to biofilm age and across biogenic zones

Microbial biofilm communities were mainly represented by bacterial taxa from four Phyla, Campylobacterota, Proteobacteria, Bacteroidota, and Verrucomicrobiota, making up 93.5%–98.0% of the microbial communities in each sandwich (Fig. 5). The taxonomic families with the highest mean relative abundances across all sandwiches included Sulfurovaceae (phylum Campylobacterota) (45.6%), Sulfurimonadaceae (phylum Campylobacterota) (18.0%), Thiotrichaceae (class Gammaproteobacteria, phylum Proteobacteria) (5.1%), Colwelliaceae (class Gammaproteobacteria, phylum Proteobacteria) (3.1%), and Crocinitomicaceae (phylum Bacteroidota) (3.1%) (Fig. 5). Overall community composition patterns suggest that both biofilm age (PERMANOVA p = 0.045) and biogenic zone (PERMANOVA p = 0.048) significantly influence the composition of microbial communities on sandwich samples (Fig. 6,Supplementary Table S4). Pairwise comparisons of community composition across biogenic zones (pairwise PERMANOVAs) identified significant differences between mussel and Alvinellid zones (p = 0.029) but not between mussel and *Riftia* zones (p = 0.12) or Alvinellid and *Riftia* zones (p = 0.33). Significant multiple linear regressions were detected between the first two ordination axes of the PCoA plot (Fig. 6, Supplementary Fig. S2) and the temperature measured at the base of each sandwich at either the start of the experiment (p = 0.024) or upon sandwich recovery (p = 0.008) suggesting that temperature (which should also be associated with biogenic zone) is an important factor in controlling microbial biofilm community composition (Supplementary Fig. S2). Since temperature is often used as a proxy for hydrothermal fluid flow, the significance of this multivariate regression may suggest that strong environmental gradients are driving microbial community composition trends resulting in the horseshoe effect (Morton et al.,

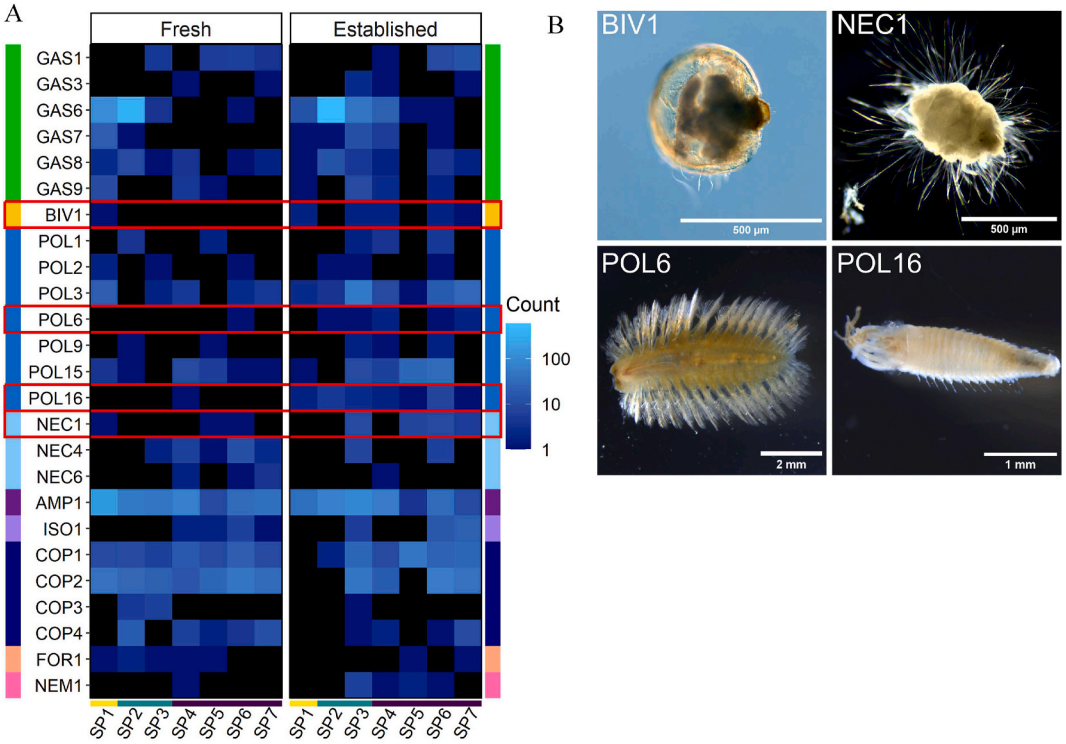

Fig. 4. A. Heatmap of faunal colonist morphotype counts (note the log-scale in the legend) across all sandwich samples separated by biofilm age (fresh vs. established). Samples are labeled by sandwich pair with colored bars on the x-axis representing the different biogenic zones (Alvinellid (yellow), Riftia (teal), Mussel (dark purple)). Morphotypes are labeled and colored based on broad taxonomic groups (see Fig. 2 and Supplementary Guide S1 for more details). Red outlines represent morphotypes that were determined to statistically differ between paired established and fresh sandwiches (p < 0.05). B. Select images of the faunal morphotypes from A that differed based on biofilm age. Top Right: BIV1 – *Bathymodiolus thermophilus,* Top Left: NEC1 – *Archinome* sp., Bottom Left: POL6 – family Polynoidae, Bottom Right: POL16 – *Amphisamytha galapagensis*.

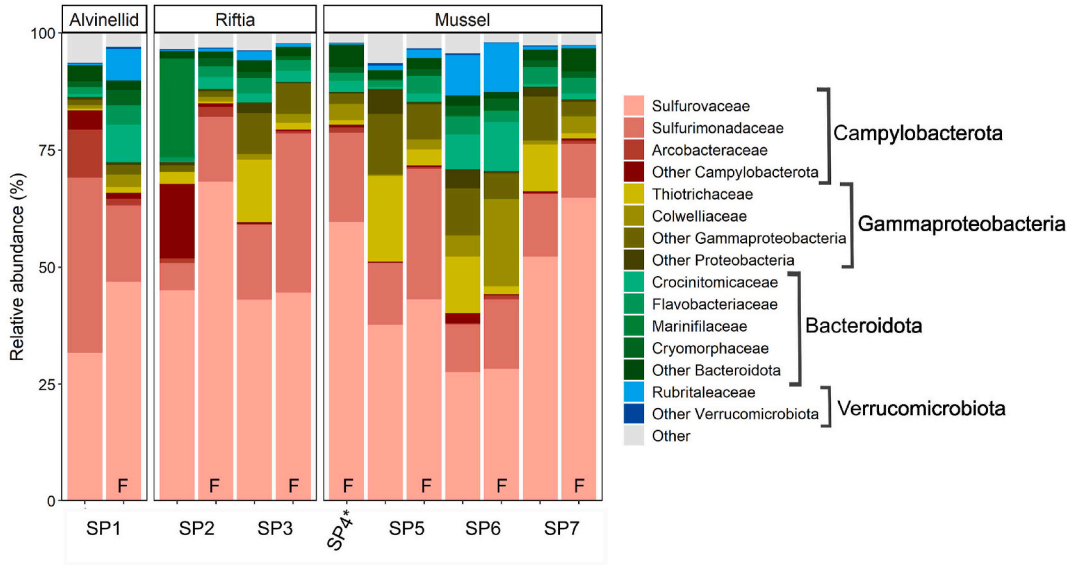

Fig. 5. The relative abundance of microbial ASVs from biofilms on each sandwich sample and colored by taxonomic family and phylum. Each sandwich pair (SP) is plotted together and grouped by biogenic zone with the fresh biofilm pair denoted with an "F". *Note the fresh sandwich pair in the mussel zone from sandwich pair 4 (SP4) did not have a sequenced established biofilm pair.

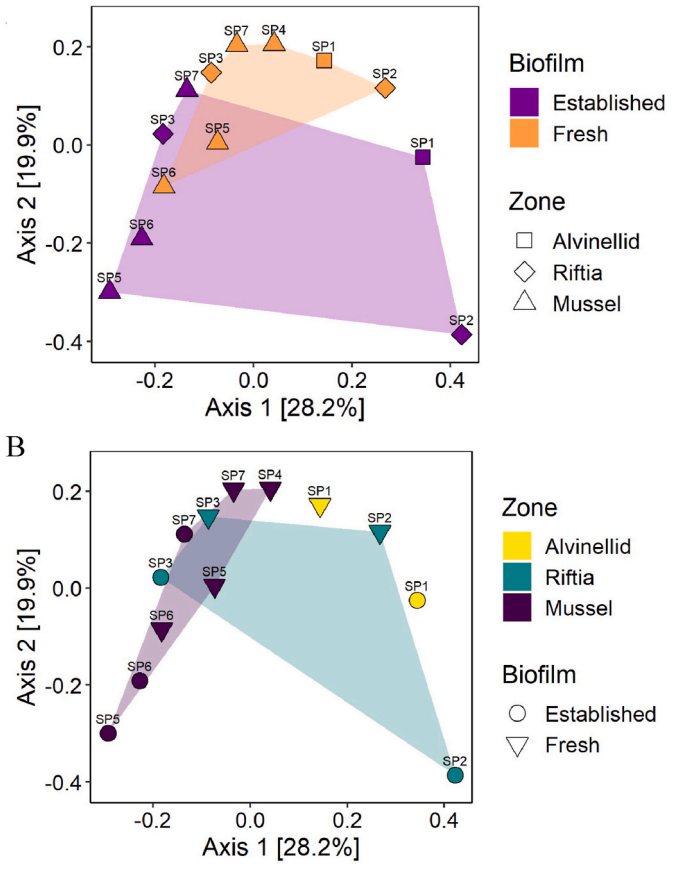

Fig. 6. Principal coordinates analysis (PCoA) of the Bray-Curtis dissimilarity matrix based on microbial ASV relative abundance in each sandwich sample. A. Sandwich samples (points) are colored by biofilm age and shaped by biogenic zone. B. Sandwich samples (points) are colored by biogenic zone and shaped by biofilm age.

2017) and the inability to fully separate communities in 2-dimensional space as shown in the ordination plot (Fig. 6,Supplementary Fig. S2).

Differences in the relative abundances of 3 of the top 10 most abundant taxonomic families were detected between paired established and fresh sandwiches (Supplementary Fig. S3). Established biofilm pairs had significantly higher Thiotrichaceae relative abundances compared to fresh biofilms (paired *t*-test p = 0.023). On the other hand, fresh biofilm pairs had significantly higher relative abundances of Sulfurovaceae (paired *t*-test p = 0.042) and Colwelliaceae (paired Wilcoxon signed rank test p = 0.031) than the established biofilm pairs. At a higher taxonomic resolution, ANCOM-BC detected 70 ASVs that were

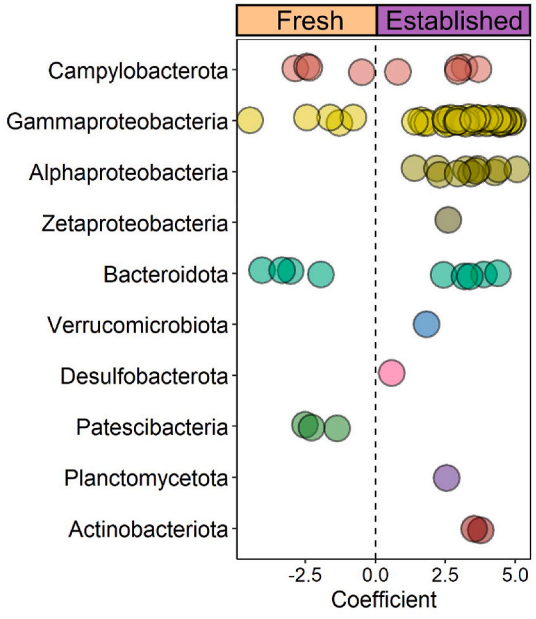

Fig. 7. Differentially abundant microbial ASVs between established and fresh biofilms accounting for biogenic zone and determined from ANCOM-BC (BH adjusted p-values <0.05). Each point represents a single ASV, if the coefficient is positive (negative), it is more (less) abundant on established biofilm sandwiches than fresh biofilm sandwiches. Points are colored based on bacterial phylum or class.

significantly enriched on established biofilm sandwiches and 16 ASVs that were significantly enriched on fresh biofilm sandwiches out of 450 total ASVs tested (BH adjusted $p < 0.05$) (Fig. 7, Supplementary Data Table S2). The ASVs whose abundance statistically differed between fresh and established biofilms belonged to eight different bacterial phyla (Campylobacterota, Proteobacteria, Bacteroidota, Verrucomicrobiota, Desulfobacterota, Patescibacteria, Planctomycetota, and Actinobateriota). Consistent with the broad family trends, 17 ASVs from the Thiotrichaceae (class Gammaproteobacteria) were enriched on established biofilms while no Thiotrichaceae ASVs were enriched on fresh biofilms. Additionally, Colwelliaceae and Crocinitomicaceae families had representative ASVs (4 and 3, respectively) that were only enriched on fresh biofilms. ASVs identified to genera *Pseudahrensia* (family Rhizobiaceae, class Alphaproteobacteria, phylum Proteobacteria), *Sedimentitalea* (family Rhodobacteraceae, class Alphaproteobacteria, phylum Proteobacteria), *Marine Methylotrophic Group 2* (family Methylomonadaceae, class Gammaproteobacteria, phylum Proteobacteria), and *HTCC5015* (family Arenicellaceae, class Gammaproteobacteria, phylum Proteobacteria) had several representatives that were enriched only on established biofilms. The three ASVs with higher abundance on fresh biofilms and belonging to Phylum Patescibacteria were all identified as unknown members of Order JGI 0000069-P22, class Gracilibacteria.

Although ASV richness and Shannon diversity varied across sandwich, biofilm age and biogenic zone were not found to significantly influence these alpha diversity metrics (ANOVA $p > 0.05$, paired *t*-test $p > 0.05$) (Supplementary Fig. S4, Supplementary Table S5-S6). It is important to note that due to the low number of total sandwich samples analyzed (13 total, 12 paired) the power to detect differences in alpha diversity is relatively low. All but one sandwich pair (in the mussel zone) had higher ASV richness on the established sandwich versus the fresh sandwich and all but one sandwich pair (in the *Riftia* zone) had higher Shannon diversity on the established sandwich versus the fresh sandwich pair.

3.3. Faunal colonist and microbe covariance patterns

Proportionality analysis revealed 51 total covariances with 17 positive and 34 negative covariances ($|\rho| > 0.6$) between microbial ASVs and faunal colonists representing potentially interacting taxa (Fig. 8, Supplementary Data Table S3). Potential settlers including *B. thermophilus* post-larvae, *Ophyrotrocha* sp. nectochaetes, and an unknown nectochaete morphotype (putatively *A. galapagensis* nectochaete) covaried with microbial taxa from Families Sulfurovaceae, Thiotrichaceae, and Saprospiraceae. Positive covariances were detected between *B. thermophilus* post-larvae and a bacterial ASV identified as *Cocleimonas* sp. (Family Thiotrichaceae) and between NEC6 nectochaetes (putatively *A. galapagensis*) and three different bacterial ASVs identified as *Sulfurovum* sp. (family Sulfurovaceae). Several immigrant faunal taxa including isopods, copepods, polychaetes, gastropods, and foraminifera also covaried with a variety of microbial taxa. Isopods had the highest number of interactions (20), mostly negative covariances with Sulfurovaceae ASVs while *Abyssotherma pacifica* foraminifera had 8 positive covariances with microbial ASVS, various gastropods and polychaete morphotypes covaried (7 covariances each) with diverse microbial ASVs, and copepods from the Dirivultidae family positively associated with two *Rubritalea* sp. (family Rubritaleaceae). Gastropod immigrants including *Bathymargarities symplector* and *Lepetodrilus* sp. exclusively negatively covaried with microbial taxa from Sulfurovaceae, Thiotrichaceae, and Ectothiorhodospiraceae Families.

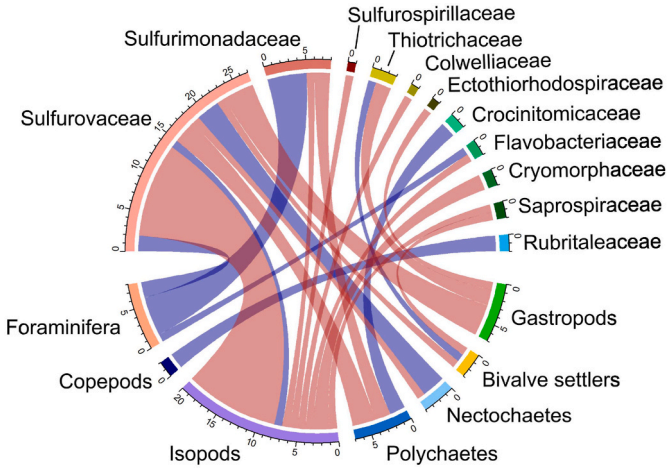

Fig. 8. Potential interactions between faunal colonists (bottom) and microbes (top) determined from proportionality analysis. The width of each segment represents the total number of covariances occurring between faunal colonist morphotypes (grouped and colored by broad morphotype group, as in Fig. 2) and microbial ASVs (grouped and colored by Family, as in Fig. 5). The lines connecting faunal colonists to microbes are colored based on whether the covariance is positive ($\rho > 0.6$, blue) or negative ($\rho < -0.6$, red).

Comparisons between the ASVs within this dataset and published 16S rRNA gene sequences from symbionts of *B. thermophilus*, *R. pachyptila*, and *A. pompejana* detected potential symbiont matches from the biofilms here. For *B. thermophilus*, a single ASV identified as belonging to the SUP05 cluster (family Thioglobaceae, class Gammaproteobacteria) had a 99.6% match to the gill endosymbiont sequence (only 1 nucleotide mis-match). This ASV (ASV284) was present in all sandwich biofilm samples with a maximum relative abundance of ~0.14% but it was not detected as differentially abundant between established and fresh biofilm sandwiches (Supplementary Data Table S2) and there was not a significant correlation between *B. thermophilus* post-larvae clr transformed pseudo-counts and ASV284 clr transformed pseudo-counts (spearman $\rho = 0.09$, $p = 0.767$). The symbiont sequences from *R. pachyptila* and *A. pompejana* were found to match ASVs here at best with 97.2% similarity (6 nucleotide mismatches and gaps) and 96.8% similarity (8 nucleotide mis-matches), respectively. The best ASV match to the *R. pachyptila* symbiont, identified as *Sedimenticola* sp. (class Gammaproteobacteria), was detected in only a single sandwich sample (fresh biofilm in the *Riftia* zone) at a relative abundance of ~0.007% while the best match to the *A. pompejana* symbiont, identified as *Sulfurovum* sp. (phylum Campylobacterota), was only detected in 2 samples (both fresh biofilms in the mussel zone) at a maximum relative abundance of ~0.007%.

4. Discussion

It is well recognized that patterns of animal colonization at hydrothermal vents are influenced by the variable and extreme abiotic conditions associated with the venting of local hydrothermal fluids (Galkin and Goroslavskaya, 2008; Gollner et al., 2010, 2015; Mullineaux et al., 2012). The results here highlight the importance of biological, specifically microbial, drivers of animal colonization patterns near hydrothermal vents. The finding that faunal community composition differed based on biofilm age across all biogenic zones suggests that differences in microbial community composition, biomass, and other biofilm characteristics, plays a significant role in larval settlement processes and the relocation of juvenile or adult animals. Thus, the abiotic environment and competition for newly opened space were not the only factors that controlled faunal colonization patterns. Since overall colonist counts were generally higher on established biofilm pairs and both potential larval settlers and presumed immigrant polychaetes had increased abundances associated with established biofilm sandwiches, there are likely several ways in which microbial biofilms interact with faunal communities including via settlement cues, trophic processes, and symbiotic relationships. It has long been hypothesized that microbial biofilms within hydrothermal vent ecosystems provide cues that animals

can interpret to locate and successfully colonize habitats that accommodate their physiological tolerances and nutritional needs (Adams et al., 2012; O'Brien et al., 2015), but this is the first study to test and confirm this using *in-situ* colonization experiments. These results focus on colonization patterns within well-established communities and do not describe how animals colonize newly created habitats after catastrophic disruptions such as seafloor eruptions, but they suggest that microbes likely influence primary colonization and further work would be necessary to assess the role of microbial biofilms on pioneer colonists at hydrothermal vents. Although there is still much to learn, these findings have considerable repercussions for the overall understanding of the ecology of hydrothermal vent ecosystems and the recovery and resilience of these systems in response to disturbances.

4.1. Established microbial biofilms enhance *B. thermophilus* and *Archinome sp.* settlement

The increased abundances of potential larval settlers including *B. thermophilus* and *Archinome* nectochaetes on established biofilm sandwiches may indicate that the presence and abundance of specific microbial taxa and/or physical or chemical properties of the established biofilms (reviewed in Hadfield, 2011; Dobretsov and Rittschof, 2020) are providing settlement cues for these faunal taxa. Many different types of invertebrate larvae are responsive to microbial biofilms but the microbial taxa that induce or inhibit settlement and the mechanisms of induction are known to vary (Hadfield, 2011; Dobretsov and Rittschof, 2020). The established biofilm sandwiches here represented a more developed bacterial community likely containing higher cell densities, biomass, and metabolic diversity than fresh biofilms (Chung et al., 2010; Gulmann et al., 2015; Sushmitha et al., 2021) (Supplementary discussion) but overall, the microbial communities making up both established and fresh biofilms were dominated by members of the Campylobacterota phylum (formerly known as Epsilonproteobacteria) and the Gammaproteobacteria class. Differences in the relative proportions of taxonomic groups and abundances of individual ASVs between fresh and established biofilms were observed, but the most abundant families and genera were not completely replaced by different taxonomic groups over time. This is in agreement with previous observations at the EPR where early microbial colonizers persisted in biofilm communities over time and appeared to have a significant influence on the development of the more established community (Gulmann et al., 2015). This may suggest that important microbial taxa are established within biofilms early, but their densities may not be high enough to act as an effective settlement/metamorphosis inducer until the biofilm has aged. Indeed, experimental evidence suggests that settlement and metamorphosis induction strength correlate with higher bacterial cell densities in both natural and mono-specific biofilms (Unabia and Hadfield, 1999; Huang and Hadfield, 2003; Lema et al., 2019).

For some larvae, settlement and metamorphosis may depend on physical contact with the biofilm surface. Indeed, for the model biofouling polychaete, *Hydroides elegans*, settlement appears to depend on surface contact and specific bacterial genes and structures have been identified to induce settlement or trigger metamorphosis (Hadfield, 2011; Huang et al., 2012; Hadfield et al., 2014; Shikuma et al., 2014, 2016). Several chemical compounds produced by bacteria within biofilms such as tetrabromopyrrole (Tebben et al., 2011; Siboni et al., 2012; Sneed et al., 2014), the quorum sensing molecules acylated homoserine lactones (AHLs) (Tait and Havenhand, 2013), and polysaccharides, proteins, glycolipids, hydrocarbons, and fatty acids within the EPS (Steinberg et al., 2002; Hung et al., 2009; Ganesan et al., 2012; Hadfield et al., 2023) have also been suggested to induce metamorphosis and settlement in various invertebrate species. In complex natural biofilms it is difficult to identify the specific taxa or chemical compounds that are responsible for cuing larval settlement, but the microbial taxa found to covary with potential faunal settlers or enriched on established biofilms may help identify potential key microbes in larval settlement processes at hydrothermal vents.

Settlement by invertebrates that host endosymbionts as adults may also be induced by free-living symbionts in the environment (Winkler et al., 2015). Indeed, the highly abundant Campylobacteria and Gammaproteobacteria groups observed here have many representatives that are found in association with chemosymbiotic metazoans at hydrothermal vents (Nakagawa and Takai, 2008). Although potential microbial symbionts of the foundation species *A. pompejana* and *R. pachyptila* were detected in biofilms here, they were found at very low prevalence and relative abundance and so microbial biofilms may not be important environmental sources of symbionts for these animals or the biofilms analyzed here may not have been developed in suitable conditions for the symbionts. For the foundational chemosymbiotic mussel, *B. thermophilus*, that relies on sulfur-oxidizing symbionts (class Gammaproteobacteria) for the bulk of its nutrition but can also acquire food through filter feeding (Page et al., 1991; Raulfs et al., 2004; Ponnudurai et al., 2017), an ASV (SUP05 cluster, class Gammaproteobacteria) closely related to a published adult *B. thermophilus* gill symbiont was present in all biofilms. Although, this ASV was not differentially abundant between fresh and established biofilms and it did not covary with *B. thermophilus* settlers. Since very few *B. thermophilus* settlers were detected on the colonization surfaces and the amplicon sequencing data cannot provide absolute bacterial abundance measures, this may still indicate that the symbiont absolute abundance or presence is important, but it is likely that other aspects of microbial biofilms also influence *B. thermophilus* settlement.

Bathymodiolus thermophilus settler abundance was found to positively associate with an ASV identified as *Cocleimonas* (family Thiotrichaceae), possibly indicating that this bacterium is important for the settlement of larval mussels at the EPR. The genus *Cocleimonas* is not well described and to date, just a single heterotrophic, sulfur-oxidizing strain has been isolated from the internal tissue of a shallow water sand snail in the Sea of Japan (Tanaka et al., 2010). Other bacteria closely related to *Cocleimonas* have been detected in the gill and gut of the hydrothermal shrimp *Rimicaris chacei* (Apremont et al., 2018), associated with the shell and scales of the scaly-foot snail (Bai et al., 2021), as an epibiont of a hydrothermal vent squat lobster (Watsuji et al., 2018), as gill symbionts of various other hydrothermal vent invertebrates (shrimps, crabs, and snails) (Lee et al., 2021), and on biofilms near hydrothermal vents (O'Brien et al., 2015; Patwardhan et al., 2018). Further work is necessary to elucidate this potential interaction between *B. thermophilus* settlers and *Cocleimonas* as it is unknown whether *Cocleimonas* may form symbioses with *B. thermophilus* or if specific cues may be produced by these bacteria that influence settlement and metamorphosis.

Not much is described about the early life history of *Archinome* polychaetes but due to the widespread occurrence of microbial biofilm induction of larval settlement, it is possible that established biofilms provide settlement cues for these larvae. Proportionality analysis did not detect significant covariances between *Archinome* nectochaetes and any microbial ASVs, but some of the enriched ASVs on established biofilms may be important for larval settlement. Indeed, several ASVs belonging to the Rhizobiaceae family were enriched on established biofilms and in terrestrial plant and soil systems, Rhizobiaceae bacteria have diverse quorum sensing systems, many of which involve AHL synthesis and perception (Sanchez-Contreras et al., 2007). Rhodobacteraceae ASVs were also enriched on established biofilms and have been implicated in AHL production and quorum sensing (Wagner-Döbler et al., 2005; Huang et al., 2008; Doberva et al., 2017). Several putative methane-oxidizing bacterial ASVs identified as Marine Methylotrophic Group 2 (family Methylomonadaceae) were also enriched in established biofilms. This bacterial group is found to associate with a variety of hosts including ciliates (Pasulka et al., 2017), squat lobsters (Watsuji et al., 2010), shrimp (Hui et al., 2022), and sponges (Rubin-Blum et al., 2019) from methane seeps or hydrothermal vents and therefore these bacteria may be important for arriving colonists. It is also important to note that the association between fauna and microbes is not unidirectional and

potential associations between faunal colonists and bacteria may indicate that colonists are transporting these microbes (and others) onto biofilms.

4.2. Specific bacterial taxa may induce larval settlement independent of biofilm age

In addition to potential settlers with increased abundance on established biofilms, other faunal settlers may also respond to characteristics of microbial biofilms that are not dependent on biofilm age. For example, putative *A. galapagensis* nectochaetes did not have increased abundances on established biofilms compared to fresh biofilms, but they did positively covary with several *Sulfurovum* ASVs, which may suggest that these bacteria play a role in settlement and metamorphosis of *A. galapagensis*. *Sulfurovum* spp. (family Sulfurovaceae, phylum Campylobacterota) have been shown to be successful initial colonizers of surfaces at hydrothermal vents (Alain et al., 2004; Gulmann et al., 2015; Patwardhan et al., 2018) which is consistent with the higher relative abundance of Sulfurovaceae ASVs (mainly identified as *Sulfurovum*) on fresh compared to established biofilm pairs observed here. The initial success of *Sulfurovum* representatives might be attributed to having genes related to biofilm production including exopolysaccharide synthesis and quorum sensing (Pérez-Rodríguez et al., 2015; Wang et al., 2023). These bacterial genes and the metabolites produced by *Sulfurovum* may also play a role in cuing larval settlement. Although Sulfurovaceae became proportionally less abundant in established biofilms while Thiotrichaceae (class Gammaproteobacteria) became proportionally more abundant, *Sulfurovum* ASVs and the Sulfurovaceae family remained dominant in both fresh and established biofilms. Various other prominent microbial taxa with diverse metabolic and biogeochemical functions within the biofilms may also influence fauna colonizing these surfaces (Supplementary discussion).

4.3. Other interactions between microbial biofilms and faunal colonists

The faunal colonist communities analyzed here included many taxa or groups that have been commonly observed in other studies at the EPR (e.g., Micheli et al., 2002; Mullineaux et al., 2003, 2012, 2020; Govenar et al., 2005; Mills et al., 2007; Galkin and Goroslavskaya, 2008; Gollner et al., 2010, 2015, 2020; Dykman et al., 2021). Specific comparisons to previous studies of faunal communities at the EPR are discussed further in the supplementary discussion. Many of the most abundant taxa including, *Lepetodrilus elevatus*, *Ventiella sulfuris*, dirivultid and harpacticoid copepods, *Ophryotrocha akessoni*, and *Archinome* sp. are juveniles or adults of macro- and meiofauna that are either deposit feeders or grazers that feed on free-living microbes or other small invertebrates (Govenar, 2012; Govenar et al., 2015; Chapman et al., 2019; Dykman et al., 2021). Therefore, trophic interactions between microbes and fauna are likely significant drivers of faunal colonization patterns observed here. Some of the most abundant microbial taxa in the biofilm communities here are putative chemolithoautotrophs (*Sulfurovum* (family Sulfurovaceae), *Sulfurimonas* (family Sulfurimonadaceae), unknown Thiotrichaceae) that are able to utilize a variety of reduced chemicals in hydrothermal fluids as electron donors (e.g., H_2, H_2S, $S_2O_3^{2-}$, S^0) to conserve energy for primary production (Sievert and Vetriani, 2012; Ding et al., 2017; Patwardhan et al., 2018; Zeng et al., 2021; McNichol et al., 2022). These primary producers represent an efficient and concentrated food source for grazers. Since many fauna found here have been suggested to feed on microbial prey or particulate organic material and have mobile lifestyles (Govenar et al., 2005; Chapman et al., 2019; Dykman et al., 2021), it is likely that the higher overall abundances of colonists, mainly immigrants from the surrounding area, associated with established biofilms is due to the increased food availability within the established biofilms and the rapid response of mobile animals. Increased abundances of juvenile or adult *A. galapagenis* and a Polynoidae polychaete morphotype on established biofilm sandwiches may suggest that these polychaetes rely on specific prey items associated with the established biofilm or that they are more dependent on the biofilm than other faunal colonists. *A. galapagensis* lives in mucus lined tubes and has been suggested to feed on particulate matter, including bacteria and fecal pellets, that settle on surfaces (Zottoli and Zottoli, 1983; McHugh and Tunnicliffe, 1994). They may benefit from a surface with excess detrital and bacterial biomass that has built up over time on the established biofilm. The excess food source provided by established biofilms may also have indirect effects on higher trophic levels such as carnivorous polynoids (Chapman et al., 2019; Dykman et al., 2021) that can follow immigrant grazers and deposit feeders onto biofilmed surfaces. Although there is still much to learn about trophic processes at hydrothermal vents, it is clear that microbial biofilm communities are important components of the hydrothermal vent food web where they likely impact the colonization and distribution of faunal communities.

Biofilms may interact with faunal colonists in myriad other ways in hydrothermal vent ecosystems. Due to the various microbial metabolisms within the biofilm matrix, microbes may detoxify the surrounding area via uptake and transformation of chemicals within hydrothermal vent fluids thus providing a more tolerable habitat for animals to live. Indeed, within microbial biofilm communities it has been suggested that the formation of biofilms and the metabolic functions of early colonizers can create habitable conditions for new microbial colonizers. The observed shift from Campylobacterota to Gammaproteobacteria as the biofilm ages has also been documented at shallow water hydrothermal vents and may relate to differential sulfide tolerances (Patwardhan et al., 2018). As early colonizers, *Sulfurovum* spp. were thought decrease sulfide concentration within the biofilm matrix therefore conditioning the environment for less tolerant taxa such as Gammaproteobacteria related to *Thiomicrospira* (family Piscirickettsiaceae) and *Thiotrix* (family Thiotrichaceae) (Patwardhan et al., 2018). The biofilm matrix itself may also bind metals or other toxic compounds making them less available to animals and microbes (Sievert and Vetriani, 2012). Additionally, the physical texture of the biofilm may allow for firmer attachment of animals to surfaces (Zardus et al., 2008) and this may have been important for *A. galapagensis* juveniles/adults or *B. thermophilus* settlers that are generally less mobile and attach via tubes or byssal threads to surfaces. It is also important to note that the colonization patterns observed here were within already established vent communities with actively interacting microbes and fauna. From the results here, it is not possible to fully disentangle biotic interactions between microbes and fauna from biotic interactions among fauna. Because there were differences in the immigrant colonists on the sandwiches, it is still possible that the presence or abundance of immigrant colonists either directly or indirectly impacted potential settlers. However, the short-term nature of our experiment limited the likelihood of faunal facilitation of settlement.

4.4. Influence of biogenic zone on faunal and microbial communities

The finding that biogenic zone was an important factor in overall faunal and microbial community composition confirms the influential role of the proximity to and strength of diffuse hydrothermal fluid flow in structuring communities (Micheli et al., 2002; Mullineaux, 2013). The observed zonal community structuring was expected given that gradients of temperature and chemistry across these biogenic zones create niches that organisms fill based on their physiological tolerances. Additionally, the colonization sandwiches were placed directly into established communities with sources of local immigrants and biotic controls (e.g., predation, competition, symbiosis) already in place. Therefore, the surrounding community had a significant influence on access to the newly exposed colonization surfaces. The thermal and chemical environments of the biogenic zones targeted here have been previously characterized with the most intense hydrothermal fluid flow and the highest temperatures in the Alvinellid zone while *Riftia* and mussel zones are generally exposed to less intense fluid flow and are

characterized by temperatures less than ~30 °C and ~10 °C, respectively (Luther et al., 2001; Micheli et al., 2002; Le Bris et al., 2005; Le Bris and Gaill, 2007; Mullineaux, 2013). Here, temperature measurements, often used as a proxy for hydrothermal fluid flow, taken in association with the sandwich samples never surpassed 14 °C and therefore these experiments may not have been located across the full gradient of abiotic conditions experienced by local communities. The single-point measurements recorded just twice at each sandwich are also likely not representative of the full-range of conditions experienced by the communities over the course of the experiment. Despite this, temperatures recorded during sandwich deployment and recovery were significantly related to both faunal and microbial community ordination axes suggesting that temperature, and hence hydrothermal fluid flow, does indeed appear to be an important factor in structuring these communities. Previous work has described faunal and microbial distributions across these zones and in relation to a suite of chemical and temperature measurements (e.g., Le Bris et al., 2005; Mills et al., 2007; Matabos et al., 2008; Gollner et al., 2010, 2015). Here, detailed characterization of abiotic drivers of microbial and faunal community assembly is not possible without continuous or high-resolution measurements of temperature and chemistry but this will be a focus for future studies.

Although biogenic zone can be a good indication of local physicochemical conditions experienced by biological communities, sometimes these zones are not clearly differentiated, and they can shift over time. Additionally, abiotic conditions have been shown to vary substantially second-to-second and at sub-cm scales (Le Bris et al., 2005), making them difficult to measure and describe on broad scales, even over the area covered by a single sandwich. Since the established biofilm sandwiches were initially deployed within the targeted zones but left on the seafloor for more than a year, the surrounding biological communities and sometimes even the location of the sandwiches changed (e.g., crabs have been observed moving sandwiches). The temperature measurements taken by ROV *Jason* at the start and end of the colonization experiments suggest that sandwiches deployed in the alvinellid and *Riftia* zones were not bathed in temperatures as high as expected based on previous characterization of these communities (Micheli et al., 2002; Le Bris et al., 2005; Le Bris and Gaill, 2007; Mullineaux, 2013). This may be due to the high spatial and temporal variability that cannot be accurately detected by relatively slow-response and large sensors (Le Bris and Gaill, 2007) and/or the placement of sandwiches (not fully nestled into communities but placed on top or to the side of the target biogenic zones). Temperature measurements from conventional high-pressure probes, like those deployed by ROV *Jason* or HOV *Alvin* have been shown to significantly underestimate the variability in temperature fluctuations at the mixing interface of seawater and hydrothermal fluid (Le Bris et al., 2005; Le Bris and Gaill, 2007). Although zone was a significant factor controlling faunal and microbial community composition, the variability in community composition would likely be better described by accurate temperature and chemistry characterizations for each of the sandwich colonizers and greater replication of sandwich samples within zones. This would also allow for a better description of specific faunal and microbial taxa distributions in relation to abiotic conditions.

4.5. Conclusions and future directions

Microbial communities that capture chemical energy from the mixing of hydrothermal fluids and seawater to fix carbon through chemosynthesis form the basis of highly productive hydrothermal vent ecosystems. They are a prerequisite to recovery of these ecosystems after disturbances (Shank et al., 1998; Alain et al., 2004) and their functions have broad consequences for the local ecosystem and ocean biogeochemical cycling. The prevalence of chemosymbiosis between microbes and foundational faunal species at hydrothermal vent ecosystems highlights the tight coupling of microbial and animal communities that appears to be a key feature of life at extreme, highly disturbed, and patchy deep-sea environments. Despite this, very little research has focused on the interactions between free-living microbes and faunal communities. Here we found that patterns of faunal colonization not only depended on local abiotic factors but also on microbial biofilms developed on surfaces within diffuse hydrothermal flow. Microbial biofilms likely provide settlement cues, food resources, and various other functions that animal communities rely on for colonization and persistence in these environments and recovery from disturbances. This study highlights faunal and microbial community changes according to biofilm age and identifies potential interactions between specific faunal and microbial taxa. These findings demonstrate the need for increased knowledge of the ecology of potentially important vent animals and microbes and provide a starting point for forming hypotheses.

Although this study does not assess primary colonization after disturbances such as eruptions, it suggests that microbial communities may have significant influences on larval settlement and the recolonization of various vent animals after disturbances. Most of the larval and immigrant colonists found here were abundant in surrounding established communities, although several colonist morphotypes detected here are not well characterized. Therefore, competition and the local colonist pool likely influenced the faunal community on the settlement surfaces. These processes would likely differ in the context of a catastrophic disturbance such as an eruption where mobility and dispersal from remnant or distant communities are more important factors for colonizing pioneer species (Mullineaux et al., 2010; Gollner et al., 2020). Nevertheless, given that our results show biofilms impact colonization patterns of vent fauna, including larval settlement of foundational species, we expect the ability of pioneer colonists to assess the suitability of the habitat through interactions with microbes would be critical for successful establishment. The time is ripe to explore the interactions between microbes and fauna at hydrothermal vent ecosystems with the development of improved sampling and observation technologies and the advancement of molecular biology techniques (Danovaro et al., 2014; Dobretsov and Rittschof, 2020). Further work to explore biofilm chemical and physical characteristics, larval settlement behavior, microbial genetic potential and protein expression, trophic relationships, and general microbial and animal physiology and ecology will allow for interpretation of potential interactions and the roles of organisms within the broader community and environment.

Dedication

We dedicate this work to Diane K. Adams. Her contributions to the conceptualization of this project made it possible.

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T.M. Ladd: Writing – original draft, Visualization, Project administration, Investigation, Formal analysis. **M. Selci:** Writing – review & editing, Formal analysis, Data curation. **D.J. Davis:** Writing – review & editing, Investigation. **O. Cannon:** Writing – review & editing, Investigation. **C.Q. Plowman:** Writing – review & editing, Methodology, Investigation. **I. Schlegel:** Writing – review & editing, Investigation. **A. Inaba:** Writing – review & editing, Investigation. **S.W. Mills:** Writing – review & editing, Methodology, Investigation. **C. Vetriani:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **L.S. Mullineaux:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **S.M. Arellano:** Writing – review & editing, Supervision, Project administration, Methodology,

Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data and code used for this study are available in the supplementary materials, in the BCO-DMO repository (https://www.bco-dmo.org/project/851182), and at https://github.com/Arellano-Larval-Lab/Biofilms4Larvae_ms_Ladd2024. Microbial sequencing data are available through the National Center of Biotechnology Information (NCBI) with bioproject accession number PRJNA1061561.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsr.2024.104314.

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