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Research Article

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Combinatorial characterization of bacterial taxa-driven differences in the microbiome of oyster beds

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Abstract

Oyster reefs and beds provide crucial ecosystem services, including water filtration, coastal protection, and habitat provision for marine species. However, these habitats face significant threats from climate change and anthropogenic stressors. To address these challenges, numerous oyster restoration initiatives have been undertaken globally. Intertidal microbial communities, comprising diverse bacteria, archaea, and unicellular eukaryotes, drive key biogeochemical processes, but remain poorly understood in ecosystems targeted for oyster restoration. We investigated the microbiome associated with the Olympia oyster (*Ostrea lurida)*, a key restoration target in the western USA, by characterizing microbial abundance and diversity in sediment, oyster-associated biofilm, and oysters at four sites in San Francisco Bay. Our findings revealed distinct microbial assemblages in oysters and sediment compared to biofilm. Diversity, measured by the Shannon index, was highest at Heron's Head (5.47), followed by Brickyard Park (5.35), Dunphy Park (5.17), and Point Pinole (4.85). Notably, microbes *Ruminococcus*, *Streptococcus*, *Staphylococcus*, *Prevotella*, *Porphyromonas*, *Parvimonas*, *Neisseria*, *Lactococcus*, *Haemophilus*, *Fusobacterium*, *Dorea*, *Clostridium*, *Campylobacter*, *Bacteroides*, and *Akkermansia* were positively associated with biofilm collected from hard surfaces where oysters were attached at most sites. Potential implications of these microbial associations for oyster growth, survival, and interactions with environmental factors remain largely unexplored. Future research is warranted on culturing specific microbes to assess their functions and investigating environmental and ecological drivers of microbial diversity to determine relationships indicative of oyster health and potential for enhancing oyster recruitment.

Sustainability Statement

Our work contributes directly to several United Nations Sustainable Development Goals (SDGs) by promoting environmental stewardship, biodiversity conservation, and sustainable marine resource management. Specifically, our research is closely aligned with SDG 13 (Climate Action), SDG 14 (Life Below Water), and SDG 15 (Life on Land). By enhancing understanding of the microbiomes associated with oyster reefs, we are contributing to the preservation of vital marine ecosystems (SDG 14) and supporting sustainable approaches to coastal management and restoration (SDG 13). Additionally, microbial interactions in oyster beds play a significant role in maintaining healthy ecosystems, which aligns with efforts to restore terrestrial and aquatic biodiversity (SDG 15).

Keywords: restoration; Olympia oyster; biofilm; population; climate change

Introduction

Oysters are valuable foundation species, offering a myriad of ecosystem services (Luckenbach et al. 1999, Newell 2004, Kellog et al. 2014, Zu Ermgassen et al. 2015, Jiang et al. 2019, Yu and Gan 2021). The physical structure of oyster beds has the potential to reduce wave energy and protect shorelines from erosion, serving as an effective form of coastal defense (Scyphers et al. 2011, Morris et al. 2019, Perricone et al. 2023). Similar to other estuarine and marine habitats, oyster beds have the potential to provide habitat for diverse marine species. These biogenic structures create three-dimensional living spaces for breeding, feeding, and shelter, fostering high biodiversity while contributing to the productivity and function of estuarine ecosystems (Coen et al. 2007, Beck et al. 2011, Grabowski et al. 2012, 2022 ; Chan et al. 2022, Smith et al. 2022).

Globally, oyster reefs and beds are increasingly imperiled by a confluence of threats originating from both climate change and a range of human activities with some areas experiencing a 99% reduction from historic levels (Coen and Luckenbach 2000, Beck et al. 2011, Wilberg et al. 2011, Waldbusser et al. 2015). In a recent global review, Okon et al. (2023) highlight how aquatic pathogens promoted by climate change will likely affect oysters. Additionally, alterations in ocean chemistry due

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to nutrient runoff (Dame 1996) and increased carbon dioxide absorption can compromise oysters' ability to build and maintain calcium carbonate shells, a process critical for survival (Gledhill et al. 2015).

Additionally, rising temperatures and sea-level rise pose significant threats to oyster beds by altering habitat conditions and increasing thermal stress (Heo et al. 2023). In response to these challenges, numerous oyster restoration initiatives have emerged, showcasing a concerted effort to preserve and restore these vital ecosystems (Coen et al. 2007, Beck et al. 2009, Zu Ermgassen et al. 2016, Wasson et al. 2020). These efforts often involve collaborations among scientists, resource managers, and stakeholders, employing strategies from reseeding programs to the installation of artificial substrates designed to mimic natural oyster beds (Brumbaugh and Coen 2009, Schulte et al. 2009, Boudreau and Worm 2012, Baggett et al. 2015, Ridlon et al. 2021, Perricone et al. 2023).

A significant, yet poorly understood, component of oyster reefs and beds is their microbial communities. Microbiomes form a substantial proportion of biofilm and drive cycling of nutrients and transformation of materials through key biogeochemical processes (Dame 1996, Dang and Lovell 2016, Freeman et al. 2016, Remple et al. 2021, Mannochio-Russo et al. 2023). Particularly, the environmental microbiome is likely to play a crucial role in supporting the health and resilience of oyster populations. Diner et al. (2023) demonstrated the influence of environmental variables on pathogen accumulation in oysters. Destoumieux-Garzón et al. (2024) summarize knowledge on oyster–microbiota interactions for key physiological processes, including digestion, immunity, and possibly even adaptation to heat stress, thus implying an intricate connection between microbial communities and oyster resilience (Newton et al. 2007, Acevedo-Whitehouse and Duffus 2009, Trabal et al. 2012, Trabal Fernandez et al. 2014, Sakowski et al. 2020).

Despite their importance, to our knowledge, the complexity of microbial communities and their interactions with environmental factors and oysters is less understood. Enhanced understanding might inform restoration efforts, promoting environmental conditions that support both the oysters and their associated microorganisms, which collectively facilitate the functioning and persistence of these vital habitats (Baggett et al. 2015, Lokmer and Wegner 2015).

In our study, we sought to shed light on the microbiome within and surrounding the Olympia oyster (*Ostrea lurida*), the only native oyster species on the West Coast of North America, by characterizing microbial abundance and diversity in sediment, biofilm, and oyster tissue collected from four ecologically distinct sites in San Francisco Bay. These sites were selected due to their relevance to oyster restoration and their contrasting environmental conditions—including substrate type, hydrodynamic exposure, proximity to freshwater inputs, and presence of habitat features like eelgrass and macroalgae, which likely shape microbial community composition. Understanding these spatial patterns provides insights into site-specific microbial dynamics, which may inform future restoration strategies.

In this study, we examined three distinct sample types—oyster tissue, biofilm, and sediment—to characterize microbial communities within and around Olympia oyster habitats in San Francisco Bay. Oyster tissue samples were analyzed to investigate the oyster-associated microbiome, which plays a role in digestion, immune defense, and overall health (King et al. 2012). Sediment samples provided insights into the baseline microbial communities in the surrounding environment, which may influence oyster-associated microbiota through sediment–water interactions (Harris et al. 2016). Biofilm samples were collected from hard surfaces where oysters attach, including oyster shells and restoration substrates, representing the microbial communities that colonize these surfaces and contribute to biofilm-mediated ecological processes (Heo et al. 2023). Biofilms are complex microbial assemblages that play a crucial role in marine ecosystems, influencing nutrient cycling, habitat formation, and species recruitment (Harris et al. 2016). These microbial layers facilitate larval oyster settlement, regulate biofouling dynamics, and may impact oyster health by harboring beneficial or pathogenic microbes (Heo et al. 2023). However, the microbial composition of biofilms within oyster habitats remains underexplored, particularly in dynamic estuarine environments such as San Francisco Bay.

We utilized 16S rRNA gene sequencing to provide a comprehensive profile of the microbial communities across different components of the oyster ecosystem (Caporaso et al. 2012, Burgess et al. 2017) to answer the research question—how does the microbiome within and surrounding the native intertidal oyster vary spatially within the Bay? Specifically, we hypothesize that (1) microbial composition will differ significantly among sample types (oyster tissue, sediment, and biofilm), with sediment exhibiting the greatest microbial diversity due to its role as a microbial reservoir; (2) oyster-associated microbiomes will be distinct from surrounding environmental microbiomes (sediment and biofilm), likely due to host-specific microbial selection; and (3) biofilm microbial communities will differ from sediment communities, owing to their function in surface colonization and potential oyster recruitment. To this end, sediment samples were analyzed to understand the baseline microbial communities associated with sites where oysters are present, while biofilm samples were used to assess the microorganisms directly associated with the hard surfaces on which oysters live—which may influence oyster health and nutrient cycles, as has been found with another oyster species (Harris et al. 2016). Oyster tissue samples were also collected and analyzed to investigate the gut microbiome, which contributes to oyster digestion and immune function (King et al. 2012). Although environmental factors likely interact with the microbiome, this was not within the scope of the present study. For the purpose of this study, 16S rRNA amplicon gene sequencing data were analyzed and presented, representing the first attempt to unravel the microbial dynamics of native intertidal oyster beds in San Francisco Bay.

Materials and methods

Site description

The sampling strategy included a systematic collection of oysters, sediment, and biofilm from four sites (Point Pinole 37.992163, −122.356120; Brickyard Park 37.905182, −122.544006; Dunphy Park 37.861316, −122.485; and Heron's Head 37.738, −122.373) in San Francisco Bay in November 2023 (Fig. 1). The sites were chosen because existing oyster restoration efforts are underway in Point Pinole and Heron's Head and have been recommended for Brickyard Park and Dunphy Park (Subtidal Habitat Goals 2010). Rather

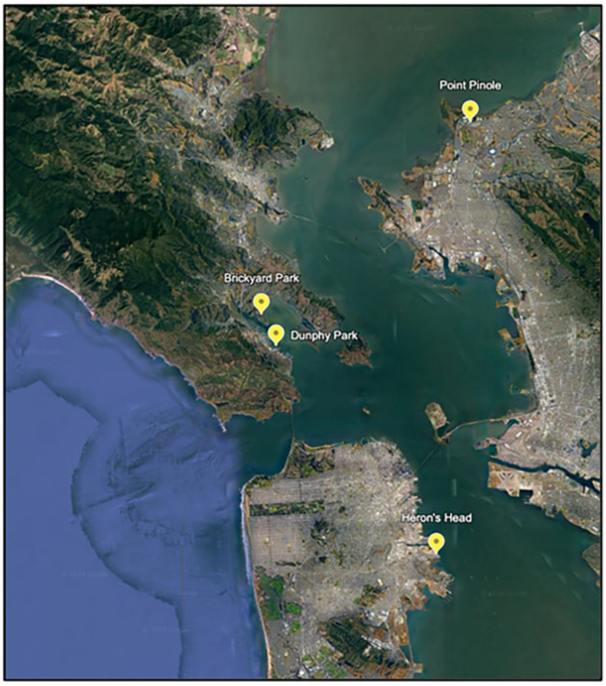

Figure 1. Study sites for oyster, biofilm, and sediment sampling in San Francisco Bay.

than focusing on a single location, this study included multiple sites to capture a broader range of natural variability in oyster-associated microbiomes, ensuring that observed microbial differences reflect patterns across intertidal oyster habitats rather than site-specific anomalies.

Each site exhibits distinct environmental characteristics that may influence microbial assemblages:

1. **Point Pinole**: Located farthest upstream, this site is characterized by significant salinity fluctuations due to its proximity to freshwater inflows. The substrate consists of mixed sand and mud, and the site has experienced oyster mortality events, particularly during the 2023 atmospheric river events. The elevation is ~1.5 m above mean sea level, and the site is exposed to moderate wave action.
2. **Brickyard Park**: Situated in Richardson Bay, this site is influenced by tidal flows and has a substrate dominated by fine sediment and eelgrass beds. The elevation is ~1.2 m above mean sea level, and the site experiences relatively stable salinity levels compared to Point Pinole. Bat ray activity, which disturbs the sediment, is frequently observed here.
3. **Dunphy Park**: Also located in Richardson Bay, Dunphy Park shares similar environmental conditions with Brickyard Park, including tidal influence and eelgrass presence. However, it has a slightly higher elevation (1.3 m above mean sea level) and a more heterogeneous substrate composition, including patches of gravel and shell debris.
4. **Heron's Head**: This site has undergone significant shoreline restoration efforts, resulting in altered sediment texture and microbial community succession. The elevation is ~1.0 m above mean sea level, and the substrate is primarily mud with occasional patches of macroalgae. The site is exposed to strong tidal currents and has a higher organic matter content compared to the other sites.

Environmental variability at these sites—including differences in substrate composition, tidal exposure, proximity to freshwater input, and presence of vegetation (e.g. eelgrass, macroalgae)—likely contributes to spatial differences in microbial assemblages. While site-specific physicochemical parameters such as nutrient concentrations and temperature fluctuations were not directly measured in this study, our results highlight the need for future work that integrates these environmental factors into microbiome analyses.

It is worth pointing out that differences in the surrounding habitat structure, environmental conditions, and/or disturbances present, such as eelgrass, macroalgae, and bat ray activity, could have affected microbial assemblages present in the sites. Additionally, given their geographical locations, the chosen sites are predisposed to harbor varying oyster densities, although this was not considered as part of this study. For instance, Point Pinole (the site that is farthest upstream) is known to host species more tolerant to salinity swings; Heron's Head has undergone major earthmoving for shoreline restoration efforts that could have impacted sediment texture and microbial community succession. The two sites in Richardson Bay (Brickyard Park and Dunphy Park) are likely to be more similar to one another ecologically compared to the other sites. Lastly, the wet season of 2023 witnessed several large atmospheric events that caused oyster mortality at Point Pinole.

Sample collection

At each site, sampling was conducted in the intertidal zone at mid-to-low tide, targeting areas where Olympia oysters (*Ostrea lurida*) were visibly present. Sampling locations within each site were selected using a random approach within a 10 m × 10 m area, ensuring representation across different microhabitats. Sediment and biofilm samples were taken from areas adjacent to oysters at a consistent elevation (~0.3–0.6 m above mean low water level), maintaining uniformity across sites. To collect sediment, three 50 ml Falcon tubes were filled with sediment from the top 10 cm from every site, following which they were placed in a sterile bag. To collect biofilm, three 15 ml Falcon tubes were first filled with 8–10 ml of 99.5% ethanol. Using a sterilized scraper, biofilm from substrates (surfaces with oysters attached to them) was scraped and placed into the ethanol-filled tube while taking care to not disturb barnacles or other sessile organisms present. Biofilm samples were scraped from hard substrates where oysters were attached, including natural rock, shell fragments, and artificial restoration materials (e.g. concrete reef structures and wooden pilings, depending on site). To collect oysters, five oysters were collected per site and placed in separate sterilized bags (WhirlPak). A total of 44 samples were collected across four sites (Point Pinole, Heron's Head, Brickyard Park, and Dunphy Park) in San Francisco Bay to assess microbiome composition in different components of the oyster ecosystem. These included 20 oyster tissue samples, 12 biofilm samples, and 12 sediment samples. All collected samples were stored in a cooler for transportation to the San Francisco State University campus, where they were subsequently stored in a −80°C freezer for downstream processing.

No human subjects or other vertebrates were involved in this research.

Sample processing

For each site, five oysters were collected and dissected under sterile conditions. At each site, oyster condition was visually assessed before collection. Observations included whether oysters were alive, dead, or showed signs of stress (e.g. gaping shells, fouling organisms, or abnormal discoloration). If oysters appeared unhealthy or were part of a mortality event, this was noted during sampling but did not influence sample selection. At Point Pinole, where oyster mortality was observed, tissue samples were collected only from live oysters to ensure the study focused on healthy microbiome communities. Whole oyster tissue was excised and pooled per site, then homogenized thoroughly following the manufacturer's protocol [DNeasy Blood & Tissue Kit (Qiagen)] for optimal DNA extraction efficiency. From the homogenized mixture, 25 mg ± 1 mg of oyster tissue was subsampled for DNA extraction to ensure uniform representation of microbial communities within each site. DNA was extracted using the Qiagen DNeasy PowerSoil Kit following the manufacturer's instructions.

Sediment processing—Upon arrival at the lab, equal volumes of sediment from the three tubes were pooled and homogenized by manual mixing in a sterile container to ensure a representative composite sample per site. From this homogenized sediment, 250 mg was subsampled for DNA extraction. DNA was extracted using the Qiagen DNeasy PowerSoil Pro Kit, following the manufacturer's instructions.

Biofilm processing—For each site, biofilm was collected using a sterilized scraper, with care taken to avoid disrupting barnacles and other sessile organisms. Biofilm was immediately suspended in 99.5% ethanol (8–10 ml) within three 15 ml Falcon tubes per site. Upon return to the lab, the contents of the three tubes were pooled and vortexed to ensure homogeneity. Due to the ethanol suspension, rather than using a 250 mg mass-based subsample, we used a 250 μl volume-based subsample for DNA extraction. DNA was extracted using the Qiagen DNeasy PowerSoil Pro Kit, following the manufacturer's instructions.

Concentration and nucleic acid extractions

For oyster tissue DNA extraction, the DNeasy Blood & Tissue Kit (Qiagen) was used following the manufacturer's instructions. 25 mg ± 1 mg of oyster tissue was used. The samples were incubated in a water bath at 56°C overnight (12+ hours). For maximum yield, the elution step was repeated as per the manufacturer's protocol. For sediment and biofilm DNA extractions, the DNeasy PowerSoil Pro Kit was used following the manufacturer's instructions. Given that the biofilm was suspended in isopropyl alcohol (99.5%) upon retrieval, instead of a 250 mg sample being used, 250 μl of the mixture was used. DNA extracts were stored in a −80°C freezer until they were shipped. DNA extracts were then sent for 16S rRNA amplicon sequencing to the Genomics Core at the University of California, San Diego. Before sequencing, the 16S rRNA V4-V5 variable region was amplified with primers 515F and 806R (Caporaso et al. 2011) using Illumina MiSeq. Owing to possible sample spoilage or DNA degradation, there were no data obtained from biofilm samples from Brickyard and oyster samples from Dunphy Park. Missing samples were excluded from downstream statistical analyses rather than imputed to avoid bias. Downstream statistical analyses using a Type III analysis of variance (ANOVA) were used to ensure valid comparisons despite unbalanced data.

Microbiome analysis

Paired-end sequences (2 × 150 bp) were processed and taxonomically classified using QIIME2 (v. 2020.6) and SILVA database (SSU r138-1) (Estaki et al. 2020, Cyphert et al. 2025). Reads were normalized using a single rarefaction step (feature count cut-off: 6993) (Mallick et al. 2017, Weiss et al. 2017). Amplicon sequence variants (ASVs) generated in QIIME2 were used to calculate alpha diversity (Shannon index; richness) and beta diversity (Bray–Curtis dissimilarity) using the vegan package (v. 2.5–7) in R (v. 4.0.1) (Dixon 2003). A principal coordinate analysis (PCoA) was carried out on Bray–Curtis beta diversity (post-rarefy), and samples were grouped relative to sample type and sampling location (95% confidence ellipses). Differentially abundant genera by sample type (positively or negatively associated relative to Point Pinole) and sampling site (positively or negatively associated relative to sediment) were determined using Microbiome Multivariate Associations with Linear Models (MaAsLin2; Mallick et al. 2021). Point Pinole was chosen as a point of comparison as it is the farthest upstream and underwent significant oyster mortality (derived from observations and reports of teams that monitored the site through the early 2024 atmospheric river events). MaAsLin output is represented in a heatmap where the intensity of association reflects −log⌉10 transformed post-hoc *q*-values. Additionally, a secondary analysis using ALDEx2 R package was carried out on ASVs generating Monte Carlo simulations of Dirichlet distributions (centered log-ratio transformed) for samples, and a generalized linear model was determined based on sample type (oyster versus biofilm; sediment versus biofilm) (Fernandes et al. 2014, Nearing et al. 2022). ALDEx2 results are represented in volcano plots where significant ASVs are indicated in the upper left and right corners ($P < .05$; magnitude of fold change $> |1|$).

Statistical analysis

All statistical analyses were conducted in RStudio (v. 1.4.1106; RStudio 2021) and relevant statistical packages. The primary goal of this study was to compare microbial diversity and composition among sample types (oyster tissue, biofilm, and sediment), rather than to assess site-specific environmental factors. Alpha diversity (Shannon index, richness) was calculated using the *phyloseq* package, and statistical significance was assessed using one-way ANOVA to compare microbial diversity across sample types (oyster tissue, sediment, and biofilm) and sample locations. This included site as a factor to account for potential spatial variability but not as the main focus of the analysis. Levene's test was applied to check for homogeneity of variances, and if violated, a Kruskal–Wallis test was used instead of ANOVA. Post-hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test for ANOVA or Dunn's test for Kruskal–Wallis, with false discovery rate (FDR) correction applied where necessary. Beta diversity was assessed us-

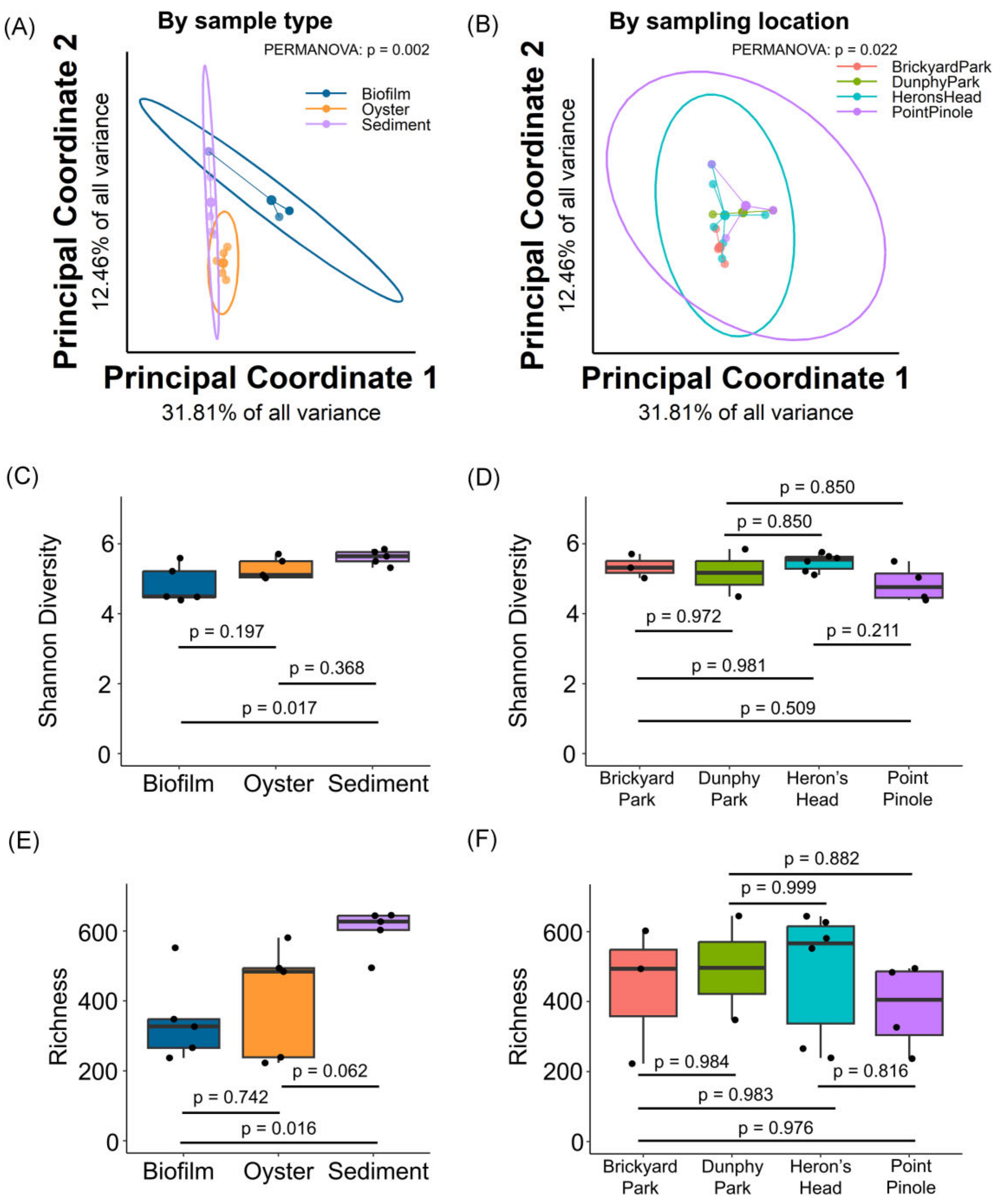

Figure 2. Differences in microbiota composition based on sample type (A) and sample location (B) using principal coordinate analysis of Bray–Curtis beta diversity. Differences in alpha diversity (Shannon diversity and richness) based on sample type (C, E) and sample location (D, F).

ing Bray–Curtis and weighted UniFrac dissimilarity indices to evaluate microbial community composition differences. PERMANOVA (permutational multivariate ANOVA) was performed using the *adonis2* function (vegan package) with 999 permutations to determine statistical significance in community composition differences across sample type and site (Anderson 2017). Pairwise PERMANOVA was conducted for significant results, with Bonferroni correction applied to adjust for multiple comparisons. To visualize beta diversity clustering patterns, PCoA was performed using *phyloseq*, and ordination plots were generated via *ggplot2*. Differential abundance analysis was conducted using DESeq2, with taxa considered significantly differentially abundant if adjusted *P*-values (Benjamini–Hochberg FDR correction) were $\leq .05$. Additionally, indicator species analysis was performed using the multipatt function (indicspecies package) to identify taxa significantly associated with specific sample types or locations. All statistical tests were conducted at a significance threshold of $\alpha = 0.05$.

Due to missing or uncollected samples at some sites, the dataset was unbalanced. To account for this, we used a Type III sum of squares ANOVA, which is appropriate for unbalanced designs as it adjusts for unequal sample sizes when testing main effects. Post-hoc pairwise comparisons were conducted using Tukey's HSD with FDR correction. The analysis was implemented in R using the car package for Type III ANOVA.

Results

Results showed 4919 unique Operational taxonomic units (OTUs) in all samples. Bacterial OTUs were assigned to 55 different phyla, while Archaea OTUs were assigned to 10 different phyla (*Crenarchaeota*, *Nanoarchaeota*, *Thermoplasmatota*, *Asgardarchaeota*, *Aenigmarchaeota*, *Altiarchaeota*, *Halobacterota*, *Hydrothermarchaeota*, *Hadarchaeota*, and *Iainarchaeota*). Across all four sites, the dominant taxa were bacteria phyla *Proteobacteria*, *Bacteroidota*, *Desulfobacteria*, *Firmicutes*, and *Cyanobacteria*. These findings are consistent with findings from literature that have utilized 16S to quantify relative abundances of individual taxa in different oyster species and geographic locations (Stevens et al. 2019). The top 20 genera in biofilm revealed the presence of animal pathogens (unclassified genera within *Enterobacteriaceae*, *Streptococcus*, *Neisseria*, *Staphylococcus*) and naturally derived microorganisms with probiotic potential (e.g. *Lactococcus*, *Akkermansia*, *Alistipes*) that resist the colonization of undesirable genera. *Sulfuvorum* and *Fusobacterium* were noted to be present in oysters that were undergoing mortality in the natural environment (Point Pinole) or getting spoiled in the refrigerator, respectively. The most dominant genus belonged to an archaeal family—*Thermoplasmatota* (*Candidatus Nitrosopumilus*).

When divided by sample type, the Bray–Curtis beta diversity showed significant differences between biofilm, oyster, and sediment samples (Fig. 2A; PERMANOVA $P = .002$, pseudo-$F = 2.86$) with the oyster and sediment having more similar compositions (indicated by the proximity of the ellipses in Fig. 2A) than in the biofilm. Microbial communities exhibited significant differences by sampling location (Fig. 2B; PERMANOVA $P = .022$, pseudo-$F = 1.78$), with Point Pinole and Heron's Head showing the greatest compositional differences. These site-level differences may be linked to distinct environmental conditions. Point Pinole, located further upstream and experiencing higher salinity fluctuations, also underwent high oyster mortality in 2023, potentially influencing microbial community composition. In contrast, Heron's Head, which has undergone shoreline restoration and receives urban runoff, exhibited microbial profiles that may reflect increased organic matter deposition. The sites in Richardson Bay (Brickyard Park, Dunphy Park) had more similar microbial assemblages, possibly due to their shared environmental features such as lower hydrodynamic activity and stable salinity. While site-specific physicochemical data were not collected in this study, these results suggest that environmental context plays a role in microbial variation and should be incorporated into future work. Shannon index and richness were the greatest in the sediment relative to the biofilm (Shannon: $P = .017$; richness: $P = .016$); differences between oysters and sediment were not statistically significant (Shannon: $P = .368$; richness: $P = .062$) (Fig. 2C, E). The maximum Shannon index indicated a high diversity in Heron's Head (5.47), followed by Brickyard Park (5.35), Dunphy Park (5.17), and Point Pinole (4.85) (Fig. 2D), but these differences were not statistically significant. Richness also indicated a similar trend (485, 440, 496, and 386 at Herons Head, Brickyard Park, Dunphy Park, and Point Pinole, respectively) (Fig. 2F). Microbial composition was significantly influenced by sample type and location, but the lack of a significant interaction effect suggests that the microbial distinctions among oyster tissue, biofilm, and sediment were consistent across sites. This indicates that site-specific environmental factors did not override the sample type-driven differences in microbial communities, reinforcing the idea that these microbial assemblages are largely structured by habitat type rather than by site-specific conditions.

Biofilm samples across sampling locations (Dunphy Park, Heron's Head, and Point Pinole) revealed intriguing ecological dynamics. Specifically, biofilm had increased *Firmicutes* and decreased *Proteobacteria* relative to oyster and sediment samples (Fig. 3). To further investigate site-specific microbial differences, we compared microbial communities at Brickyard Park and Dunphy Park relative to Point Pinole, which was uniquely affected by an atmospheric event-driven oyster die-off. Heron's Head was excluded from this comparison because previous analyses had already demonstrated that its microbial communities were significantly different from those at Point Pinole. This focused comparison allowed for a more targeted examination of microbial variation among sites that did not exhibit extreme environmental disruptions. Specifically, a combinatorial approach was used to identify differentially abundant microbes by sample type and by sampling location. MaAsLin2 analysis (Fig. 4A) identified 17 differentially abundant genera in the biofilm relative to sediment samples in which *Sulfitobacter* and *HTCC* were negatively associated with biofilm, and *[Ruminococcus]*, *Streptococcus*, *Staphylococcus*, *Prevotella*, *Porphyromonas*, *Parvimonas*, *Neisseria*, *Lactococcus*, *Haemophilus*, *Fusobacterium*, *Dorea*, *Clostridium*, *Campylobacter*, *Bacteroides*, and *Akkermansia* were positively associated with biofilm. Oyster was very similar to sediment composition with only *Sulfitobacter* being negatively associated with the oyster. Brickyard Park and Dunphy Park had one differential genus relative to Point Pinole with *Planctomycete* and *Paludibacter* positively associated, respectively.

Results from ALDEx2 differential abundance analysis (Fig. 4B) were confirmatory with 47 significant differentially abundant ASVs (41 ASVs with positive fold change, 6 ASVs with negative fold change; see taxonomic identity of ASVs—Supplementary Table 1). In the biofilm relative to the sediment, there were 80 significant differentially abundant ASVs (45 ASVs with positive fold change, 35 ASVs with negative fold change; see taxonomic identity of top 50 ASVs—Supplementary Table 2). Between the oyster and the sediment there were 16 significant differentially abundant ASVs (1 with positive fold change and 15 with negative fold changes—Supplementary Table 3). Across the sampling sites, significant differentially abundant ASVs were only detected between Brickyard Park relative to Point Pinole (eight ASVs with positive fold change—Supplementary Table 4), Brickyard Park relative to Heron's Head (five ASVs with positive fold change—Supplementary Table 5), and Brickyard Park relative to Dunphy Park (one ASV with positive fold change—Supplementary Table 6).

Discussion

Our investigation into the microbial communities associated with Olympia oyster (*Ostrea lurida*) beds in San Francisco Bay revealed complex and diverse assemblages, with distinct patterns across sample types and locations. This study was primarily guided by the hypothesis that microbial assemblages would vary spatially across the different sites we sampled from in San Francisco Bay. The results not only support this hypothesis but also provide insights into additional aspects of microbial ecology in oyster beds. Our findings suggest that

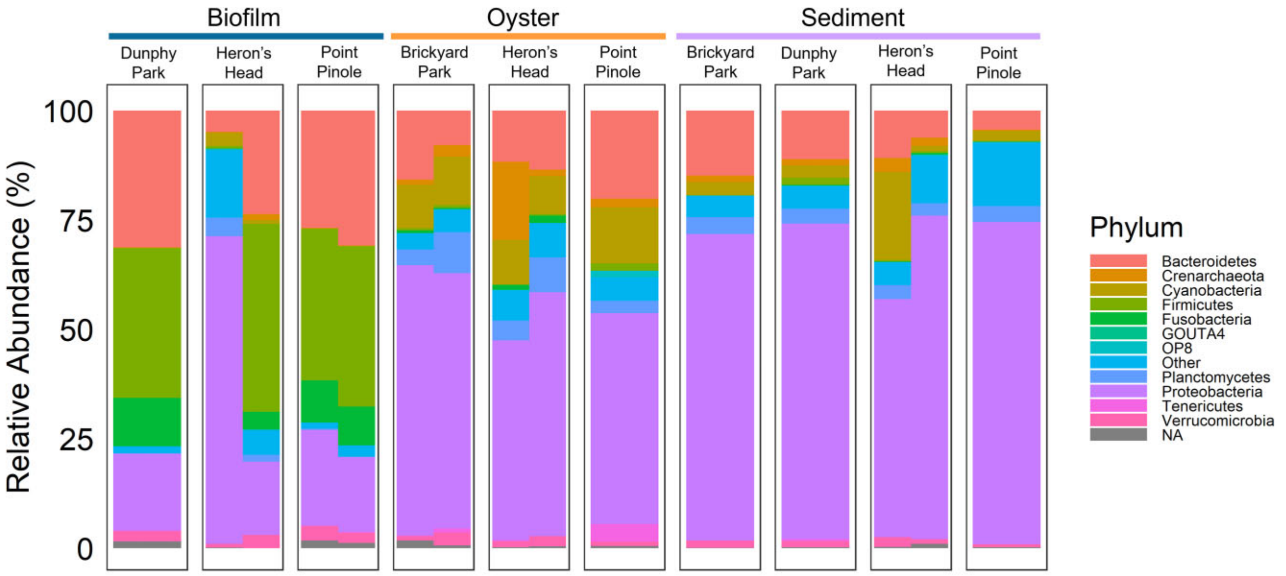

Figure 3. Relative abundance at phyla level based on sample type (biofilm, oyster, and sediment) and location (Dunphy Park, Heron's Head, Point Pinole, and Brickyard Park). Note that owing to possible sample spoilage or DNA degradation, there were no data obtained from biofilm samples from Brickyard and oyster samples from Dunphy Park. Biofilm had elevated levels of *Firmicutes* across sampling locations, and oyster and sediment had elevated levels of *Proteobacteria* across sampling locations. The steps in the bar chart indicate each individual sample. For example, the two steps for oyster from Heron's Head indicate that there were two samples as opposed to the single step for biofilm from Dunphy Park indicates that it had one sample.

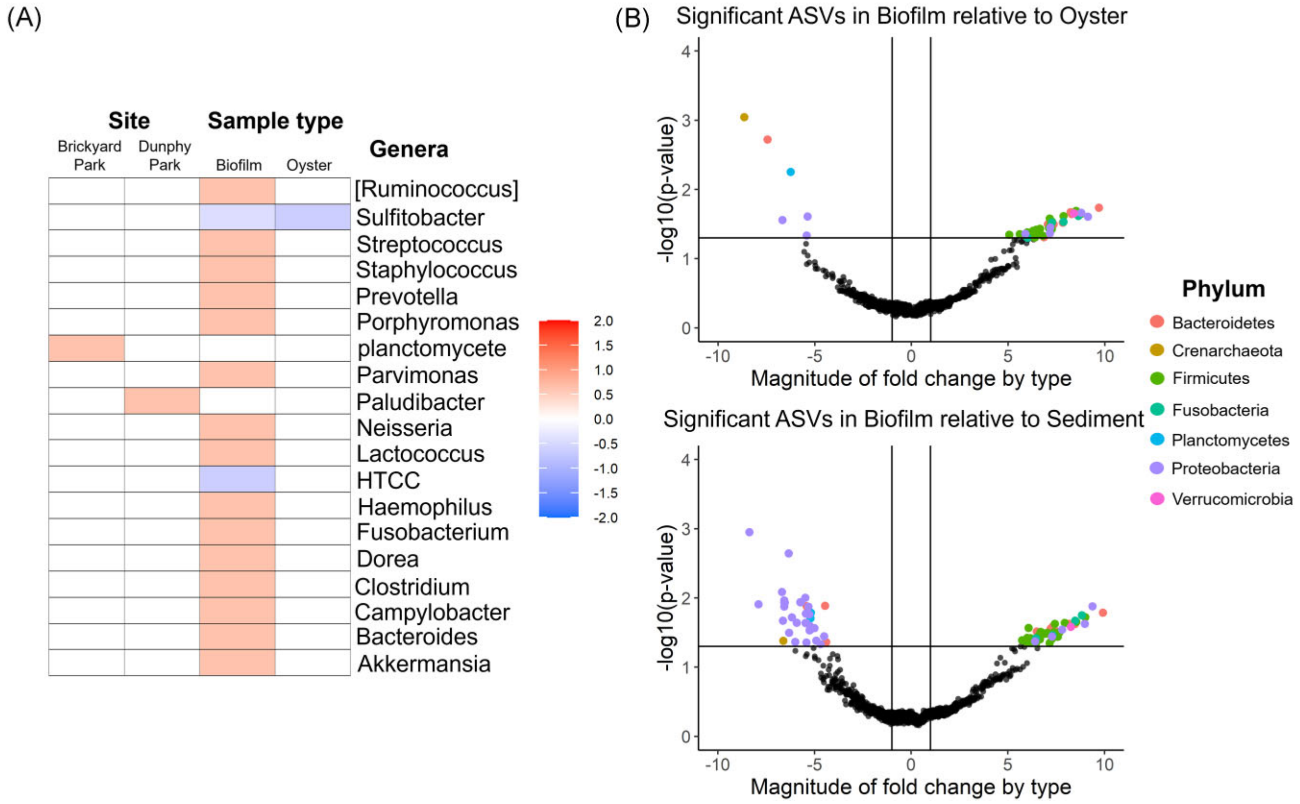

Figure 4. Differential abundance of microbes based on sample type and sampling site using two independent methods (MaAsLin2—A; ALDEx2—B). Point Pinole was selected as the reference site due to the observed oyster mortality event linked to atmospheric river disturbances. Heron's Head is not included in this comparison because it was already found to be significantly different from Point Pinole in previous analyses, making additional pairwise comparisons redundant. This figure highlights differences in microbial composition across sites that were more similar to Point Pinole, providing insights into potential environmental influences on microbial communities. Genera are positively and negatively associated with sampling site (Brickyard Park or Dunphy Park) relative to Point Pinole and associated with sample type (biofilm or oyster) relative to sediment (A). ASVs significantly increased or decreased in biofilm relative to oyster (top—B) or sediment (bottom—B). Taxonomic classification of significant ASVs at the phyla level is indicated.

1. Microbial communities exhibit significant spatial variation across different sites in San Francisco Bay, reflecting the influence of local environmental parameters. For instance, Heron's Head, which has undergone major shoreline restoration and experiences periodic low-oxygen events, had distinct microbial assemblages that may be shaped by increased organic matter input. In contrast, Point Pinole, which experienced significant oyster mortality following extreme atmospheric river events, showed lower microbial diversity, potentially reflecting disturbance-driven shifts in community structure. Sites in Richardson Bay (Brickyard Park and Dunphy Park), with their stable salinity and presence of eelgrass, exhibited more similar microbial compositions, suggesting that habitat structure plays a role in shaping microbial communities. However, because this study did not directly measure abiotic parameters (e.g. temperature, nutrient concentrations, salinity), our findings are correlative rather than mechanistic. This highlights an important next step: pairing microbial sequencing with *in situ* environmental monitoring to identify the key drivers of microbial community variation. Nevertheless, the spatial patterns observed in our study indicate that site-specific factors should be considered when designing future oyster restoration and conservation strategies.
2. There are distinct microbial assemblages associated with different sample types (biofilm, oyster, and sediment), suggesting functional specialization within the oyster beds. For instance, several microbial taxa identified in biofilm samples have potential implications for oyster health. The top 20 genera in biofilm included known animal pathogens (*unclassified Enterobacteriaceae*, *Streptococcus*, *Neisseria*, *Staphylococcus*) and naturally occurring probiotic-associated genera (*Lactococcus*, *Akkermansia*, *Alistipes*) that may resist colonization by opportunistic pathogens. The presence of *Sulfuvorum* was noteworthy, as it has been detected in oysters undergoing mortality in the natural environment (e.g. Point Pinole). The presence of *Fusobacterium* in our samples is notable, as members of this genus have been previously associated with tissue degradation and spoilage in oysters stored under refrigeration (as reported in previous studies). While our study does not provide direct evidence of spoilage, the detection of *Fusobacterium* suggests that some taxa commonly linked to oyster decomposition may also be present in live oyster microbiomes, potentially playing roles in microbial succession or opportunistic interactions. Lastly, *the most dominant genus in archaea that was detected in our samples was from the family Thermoplasmatota (Candidatus Nitrosopumilus), which has been linked to nitrogen cycling and ammonia oxidation in marine environments*. These findings suggest potential associations between microbial community composition and oyster health status.
3. The presence and abundance of certain microbial taxa may have implications for oysters and overall ecosystem health. The consistency of microbial groups across sites suggests shared functional roles in oyster-associated microbiomes rather than site-specific microbial selection. While some taxa (e.g. *Enterobacteriaceae*, *Lactococcus*, *Neisseria*) have been documented in the microbiomes of other bivalve species (e.g. *Crassostrea* spp.), further research is needed to determine whether their presence reflects active host–microbe interactions or passive environmental colonization.

These observations extend our understanding beyond the initial hypothesis, offering a broader perspective on the role of microbes in Olympia oyster bed ecosystems. The following sections explore these findings in detail, examining the physical structure and habitat preferences of microbial communities, their functional specialization, and the presence of both beneficial and potentially detrimental organisms. This discussion aims to contextualize our results within the broader framework of oyster ecology and restoration efforts in San Francisco Bay and similar coastal environments, highlighting areas that warrant further investigation.

Physical structure and habitat

The significant differences in Bray–Curtis beta diversity among biofilm, oyster, and sediment samples highlight distinct microbial community structures associated with each sample type. We hypothesize that the physical and biochemical environment of each sample type selectively enriches for different microbial assemblages. The greater similarity between oyster and sediment samples compared to biofilm suggests shared environmental conditions or an exchange of microbes between these habitats. This could be attributed to the proximity of oysters to sediment and the potential transfer of microbes through oyster feces, pseudofeces, and filter feeding of suspended particles. The differential abundance of certain microbes between locations such as *Planctomycete* in Brickyard Park and *Paludibacter* in Point Pinole indicates microbial adaptation to local environmental conditions or responses to site-specific anthropogenic effects (Diner et al. 2023). Notably, Guedes et al. (2024) demonstrated the ecological role of Planctomyceta and their antimicrobial properties, which could have implications for oyster health and restoration efforts.

Oyster mortality

We hypothesize that the observed diversity trend (Shannon index: Heron's Head > Brickyard Park > Dunphy Park > Point Pinole) may be linked to higher oyster mortality at Point Pinole, which was noted during routine monitoring and restoration efforts in San Francisco Bay (unpublished observations). While this study does not directly quantify oyster mortality, the microbial signatures observed at Point Pinole may reflect post-mortality microbial succession or shifts in microbial interactions resulting from habitat disturbance. The presence of *Sulfuvorum*, a microbial taxon previously associated with oyster mortality, further supports the idea that microbial communities at Point Pinole were influenced by environmental stressors or die-off events. However, since we did not conduct direct mortality assessments, we cannot conclusively determine the causal relationship between oyster health and microbial shifts. Instead, our findings suggest that microbial community changes at Point Pinole may serve as indicators of environmental disturbances impacting oyster populations. Lastly, although our study samples were not observed to undergo spoilage, certain microbial taxa detected (e.g. *Fusobacterium*) have previously been associated with decomposition in oysters stored under refrigeration. Their presence in this study may reflect broader microbial succession patterns rather than direct spoilage within our samples. Future research should integrate mortality assessments, environmental monitoring, and microbial functional profiling to better understand the link between extreme environmental events, oyster health, and microbial community dynamics.

Functional specialization

Building upon the observed habitat-specific microbial communities, we found increased *Firmicutes* and decreased *Proteobacteria* in biofilms within the sampled oyster beds. We hypothesize that this shift is due to unique environmental conditions in these ecosystems (Trabal Fernández et al. 2014). Firmicutes, known to thrive in anaerobic or low-oxygen environments, likely contribute to crucial nutrient cycling processes through fermentation and sulfate reduction in such ecosystems. Their presence may enhance the resilience of the microbial community against environmental fluctuations, potentially benefitting oyster bed stability. The abundance of spore-forming species, particularly within the *Firmicutes* phylum, may be triggered by the shallow anoxic layer of mud at these locations. Conversely, the decrease in Proteobacteria could be due to competition for nutrients or space, predation by bacteriophages or protozoa, or the creation of a more acidic environment by Firmicutes' fermentation processes.

Detrimental versus beneficial organisms

Our results revealed a complex interplay of potentially beneficial and detrimental microorganisms within the oyster beds. Interestingly, we observed the presence of a probiotic microbe (*Lactobacillales;* Kang et al. 2018) in the biofilm. *Lactobacillales* is known for its role as a biocontrol agent against pathogenic microorganisms (Gatesoupe 2008). Notably, a recent study by Stevick et al. (2019) demonstrated that probiotic treatment in an oyster hatchery had a systemic effect on targeted members of the bacterial community, leading to a net decrease in potentially pathogenic species. The study also identified differentially abundant microbes, with *Sulfitobacter* and other genera showing distinct associations with biofilm and sediment samples. The negative association of *Sulfitobacter* with biofilm and oyster samples suggests its adaptability or preference for conditions present in sediment. Bruhn et al. (2007) demonstrated the antimicrobial capacity of strains such as *Sulfitobacter* belonging to the *Roseobacter* clade that produce antibacterial compounds that inhibit non-*Roseobacter* clade microbes and enhance biofilm formation.

On the other hand, Brickyard Park had an increased abundance of both *Vibrio* and *Roseovarius* (both known to have some genera that are pathogenic for oysters; Boardman et al. 2008, Green et al. 2019) relative to Point Pinole and Heron's Head. The estuarine environment of San Francisco Bay, with its mix of salt and freshwater, provides a suitable habitat for *Vibrio* species, many of which thrive in brackish waters. The bay's complex water dynamics, influenced by tides and freshwater inflows, can distribute these bacteria across a wide area. Furthermore, the presence of specific genera in the biofilm, such as *Streptococcus*, *Staphylococcus*, and *Akkermansia*, highlights unique microbial signatures that may be functionally detrimental or important in these environments. For example, *Akkermansia*'s current range is restricted to aquatic environments such as the human gut and is well known for its role in breaking down mucin and other glycoproteins. Our hypothesis is that *Akkermansia* plays an important role in regulating the presence of mucus that accumulates on the oyster substrates. This could have implications for understanding biofilm-associated diseases or health, presenting a targeted area for further ecological or biomedical research.

Ammonia-oxidizing archaea

Ammonia-oxidizing archaea (AOA) are among the most ubiquitous microorganisms in the ocean, driving nitrification, nitrogen oxides emission, and methane production. Recent studies (Qin et al. 2020) have demonstrated their adaptive capacity for nutrient acquisition and energy conservation through genetic diversification associated with niche adaptation. In our study sites, we observed significantly less AOA in biofilm than in other sample types. We hypothesize that this is owing to their predominant role in nitrogen cycling by regulating the fixed forms of nitrogen species available (Martens-Habbena et al. 2015, Li et al. 2018) to other microbes present in the biofilm matrix and their adaptability to the available form of nitrogen present. AOA are more resistant to low-oxygen environments such as those in the gut of oysters and sediment, where they were also relatively more abundant than in biofilm. This suggests a vertical segregation of the AOA communities, consistent with literature (Lu et al. 2016).

These findings could be further explored for their potential in ecological monitoring and assessment, contributing to our understanding of microbial roles in ecological processes like nutrient cycling and biofilm formation. These results collectively illustrate the complex interplay between microbial communities and their environments, highlighting the potential of microbial analyses in contributing to environmental monitoring and management. Going forward, culturing specific microbes and assessing their functions as well as investigating environmental and ecological drivers of microbial diversity is warranted.

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Author contributions

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Supplementary data

Supplementary data is available at *SUMBIO Journal* online.

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Data availability

Raw V4-V5 16S rRNA DNA sequences are available at the NCBI's Sequence Read Archive Database (BioProject ID: PRJNA1108902; http://www.ncbi.nlm.nih.gov/bioproject/1108902).

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