Host and Water Microbiota are Differentially Linked to Potential Human Pathogen Accumulation in Oysters

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Abstract

Oysters play an important role in coastal ecology and are a globally popular seafood source. However, their filter feeding lifestyle enables coastal pathogens, toxins, and pollutants to accumulate in their tissues, potentially endangering human health. For example, bacterial pathogens from both marine and terrestrial sources concentrate in oysters and can cause human illness when oysters are consumed raw. While pathogen concentrations in coastal waters are often linked to environmental conditions and runoff events, these do not always correlate with pathogen

concentrations in oysters. Additional factors related to oyster hosts and the microbial ecology of pathogenic bacteria likely play a role in accumulation but are poorly understood. In this study, we investigated whether microbial communities in water and oysters were linked to accumulation of fecal indicators, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Site-specific environmental conditions significantly influenced the composition and diversity of water microbial communities, which were linked to the highest concentrations of both *Vibrio* spp. and fecal indicator bacteria. Oyster microbial communities, however, were less impacted by environmental variability and exhibited less variability in microbial community diversity and accumulation of target bacteria. Instead, changes in specific microbial taxa in oyster and water samples, particularly in oyster digestive glands, were linked to elevated potential pathogens in oysters, especially *V. parahaemolyticus*. This included an increase in cyanobacteria in both water and oyster digestive gland microbial communities, which could represent an environmental vector for *Vibrio* spp. transport and decreased relative abundance of *Mycoplasma* and other key members of the oyster digestive gland microbiota. These findings suggest that host and microbial factors, in addition to environmental variables, may influence pathogen accumulation in oysters.

Introduction

Bivalves are ecologically important animals that provide valuable coastal services and are a globally popular, potentially sustainable food source. In addition to recreational and subsistence harvesting, bivalve aquaculture operations generate numerous local jobs and approximately US$23 billion in revenue annually (1). Oysters and mussels build coastal reefs which provide habitat for coastal organisms and can prevent erosion (2, 3). Additionally, adult bivalves filter

large quantities of water to concentrate their phytoplankton prey, which can reduce nutrient loads and improve coastal water quality (3).

The ability of bivalves to thrive in highly fluctuating coastal environments can be impacted by human activities and environmental stress, impairing the provision of these ecosystem services and their commercial potential. Additionally, while concentrating their prey, many bivalves concentrate human pathogens, marine toxins, and coastal pollutants, which can endanger human health when consumed. These risks to seafood safety and security are likely to increase in the future due to global changes such as climate change, increasing human populations, pollution discharge, and rapid coastal development.

Bacterial pathogens are a major food safety concern, especially for oysters which are commonly consumed raw. Infections can be caused by fecal-borne bacteria from terrestrial sources as well as marine species, which thrive in the oyster's natural environment. For example, *Vibrio* species cause an estimated 80,000 human illnesses in the US annually and 100 deaths (4). Among these, *Vibrio parahaemolyticus* is the most common cause of infection, while *V. vulnificus* causes the greatest mortality, with 1 in 5 cases resulting in death. As these species thrive in warm conditions, they are highly concerning amidst rising global seawater temperatures (5, 6). Additionally, since *Vibrio* spp. naturally occur in marine and brackish waters, they may resist depuration processes commonly employed to remove pathogens from oysters prior to entering the food supply. Understanding the factors that cause bacterial pathogens to accumulate in bivalves is critical to preserving human and ecosystem health now and in the future.

The environmental drivers of human pathogen concentrations in coastal waters are relatively well-characterized, however, the connection between water and oyster pathogen concentrations is less clear. For fecal-borne bacteria, high concentrations in coastal waters are

69 often linked to freshwater influx and sewage spills, frequently coinciding with high concentrations
70 of nutrients and other pollutants. Marine *Vibrio* species are associated with warm water
71 temperatures and exhibit species-specific salinity distributions (reviewed in (7, 8)). They also
72 attach readily to marine particles which can enhance oyster accumulation. While the presence of
73 pathogenic bacterial taxa in the water may be a prerequisite for oyster accumulation, studies
74 investigating water to oyster pathogen transfer are either rare or yield inconsistent results,
75 suggesting that factors beyond the environment likely play a role. For example, individual oysters
76 from the same environment can have highly variable pathogenic bacterial concentrations, known
77 as the "hot oyster" phenomenon (9, 10), and associations between water and oyster concentrations
78 of pathogenic *Vibrio* species vary among studies and geographic regions. Ultimately, more
79 research is needed to examine different variables that could explain the relationships between
80 environmental factors, water to oyster human pathogen transfer, and pathogen accumulation and
81 persistence in oysters.

82 Microbial communities associated with water and oysters are understudied variables that
83 likely influence human pathogen accumulation. Microbial taxa in the water column may help
84 pathogenic species proliferate in the environment and act as vectors for uptake and concentration
85 by shellfish. For example, *Vibrio* species have been linked to phytoplankton concentrations in
86 coastal waters, and human pathogenic species have shown associations with specific
87 phytoplankton groups and individual taxa (11–15). Several of these taxa are known oyster prey
88 species. Furthermore, microbial communities associated with oyster hosts (i.e., their microbiomes)
89 could also influence pathogen accumulation if environmental factors cause changes in otherwise
90 stable microbial communities. Marine animal microbiomes often contribute to host health; critical
91 functions include food breakdown, defense against host-associated pathogens, and modulation of

92 host immune responses (Reviewed in (16–18)). In stressful conditions, however, animal
93 microbiota can shift from a health-associated state, to "dysbiosis", whereby the microbiota and
94 host no longer express beneficial synergy. This often leads to proportional increases in
95 environmental microbes that are typically rare or absent in host microbiomes, or an increase in
96 commensal host microbiota that can become pathogenic. In aquaculture organisms, host-specific
97 pathogens are well studied in this context, but the accumulation of human pathogens in animal
98 hosts as a function of dysbiosis has not been adequately investigated.

99 To investigate the influence of these understudied factors on potential human pathogen
100 accumulation in oysters, we examined fecal indicator bacteria, *V. parahaemolyticus*, and *V.*
101 *vulnificus* concentrations in water and oysters across an environmental gradient in a southern
102 California coastal bay over 4 weeks. We then applied a metabarcoding approach (16S amplicon
103 sequencing) to characterize links between these target bacteria, water and oyster prokaryotic
104 microbiomes, and environmental variables. In addition to better understanding how concentrations
105 of these bacteria in water are linked to oyster concentrations, our study aimed to characterize how
106 environmental variation influences oyster microbiomes and test whether altered host-states may
107 be linked to higher bacterial accumulation. Additionally, we sought to characterize specific
108 microbial taxa in water and oyster microbiomes that correlate with human pathogen accumulation
109 in oysters, which could indicate environmental reservoirs or vectors for pathogen transmission.
110

111 **Materials and Methods**

112 *Experimental design and sample collection*

113 Approximately 1,200 Pacific oysters were collected over a three-day period (July 31-
114 August 2, 2019) from Newport Bay, CA, then transported to holding tanks located at the Kerckhoff

Marine Laboratory in Corona Del Mar, CA within four hours of harvesting. At the Kerckhoff Marine Laboratory, oysters were pooled and arranged on perforated stacked trays in four 282.7 m^3 flow-through seawater tanks for 14 days. Seawater was first filtered through a sand filter at 15 – 20 gallons per minute and then further disinfected with a Classic UV 80-Watt Series light (Aqua Ultraviolet, Temecula, California, USA) before entering the holding tanks. Following the two-week hold time, oysters were deployed across 12 sites in Newport Bay, CA for six weeks (Figure 1). For purposes of this study, only weeks 0-4 were analyzed, however, data was collected for all 6 weeks (19). Roughly 100 oysters were deployed in 23-mm plastic mesh oyster bags at each site. At the time of oyster deployment (Week 0), 10-12 oysters were collected and processed from each holding tank to characterize composite post-depuration microbial communities in whole oysters (N=10-12) and oyster tissue samples (gills and digestive glands [DG], N=10-12 each), and water was collected at each deployment site. Thereafter, both water and oyster samples were collected at weeks 1, 2, and 4. Grab water samples were collected from each site, while oyster samples were collected as composite samples of 10 to 12 individual oysters from each site at each timepoint and for each tissue type. Water samples were stored on ice, while oysters were stored in coolers with ice packs, and transported to the Southern California Coastal Water Research Project (SCCWRP) for further processing.

Oysters were collected at 12 different sites in Newport Bay, which corresponded to different attachment substrates depending on site topography, including dock, buoy, mudflat, and seawall (Additional File 2), though all oysters were ultimately translocated to identical cages following depuration. Oysters were not observed, and thus not initially collected, from sites NBS12 and NBS13 in the back bay. The environmental variations observed at these sites (Figure 1A-C) suggest that environmental or biotic factors may have made these sites inhospitable to

138 endemic oyster growth, however low oyster populations could also be caused by illegal harvesting
139 in these areas.

140 Water samples were collected in the field in cleaned and acid-rinsed (10% HCl) 2 L
141 polycarbonate bottles. Samples were brought back to the laboratory after collection and were
142 gently mixed before filtering. Approximately 100-200 mL of water was filtered onto 0.4 µm
143 polycarbonate filters for nucleic acid extraction, which was used downstream to characterize water
144 microbial communities. Additionally, water samples were collected to measure chlorophyll *a*
145 concentration: 100 mL were collected on 25 mm glass fiber filters (Whatman) with gentle filtration
146 and stored at -80 °C until processing. Lastly, more water aliquots were used to quantify fecal
147 indicator microbial species, including *Escherichia coli,* total coliform bacteria, fecal coliform
148 bacteria, and *Enterococcus* species. Filtered samples were stored in 1.5 mL tubes and frozen in
149 Liquid nitrogen, followed by immediate storage at -80 °C until DNA extraction.

150 At SCCWRP, ~10 oysters were homogenized, and the composite was processed to quantify
151 bacterial targets according to published methods (see below and Additional File 1). An additional
152 ~10 oysters were shucked and dissected, and the gill and digestive gland tissues were separately
153 pooled and homogenized. Homogenized digestive glands and gills were stored for downstream
154 DNA extraction at -80 °C.

155 Environmental data was collected using a YSI30 Pro field instrument (Yellow Springs,
156 OH), which was used primarily to assess temperature and salinity. Filters for chlorophyll *a*
157 quantification were stored in aluminum foil in a plastic bag at -80 °C until further analysis, at
158 which point they were extracted in 100% acetone in the dark for 24 hours. Total chlorophyll *a* was
159 measured in all water samples using a non-acidification method and a Trilogy Turner Design
160 Fluorometer, as previously described (20).

161

162 *Quantification and detection of human pathogen and indicator species and OSHV-1* virus

163 The bacterial targets quantified in this study and the associated analysis methods are listed
164 in Additional File 1. For bacteria enumeration in oyster tissues, we analyzed the fecal indicator
165 bacteria (FIB) *Escherichia Coli* and fecal coliform, following approved methods developed by the
166 FDA and Interstate Shellfish Sanitation Conference (Additional File 1). Briefly, fecal coliform and
167 *E. coli* concentrations were determined by conventional five-tube multiple dilution most-probable
168 number (MPN) procedure. Lauryl tryptose broth (Difco) was utilized for the presumptive growth
169 media, with confirmation performed by inoculating liquid EC-MUG media (Difco) at 44.5 °C for
170 24 ± 2 hours. Grab water samples were processed for cultivable *Enterococcus*, *E. coli*, and fecal
171 coliform according to standard methods: EPA Method 1600, EPA Method 1603, and SM 9222-D
172 (21–23).

173 For *Vibrio* spp. targets, *Vibrio parahaemolyticus* and *V. vulnificus* were quantified using a
174 culture based MPN method. Briefly, CHROMagar *Vibrio* (CHROMagar, Paris, France) media
175 plates were prepared according to manufacturer's instructions and used to enumerate potentially
176 pathogenic *Vibrio* species. *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) concentrations were
177 determined by counting visible pink and blue colonies on the CHROMagar *Vibrio* media,
178 respectively, and adjusting for dilution (24). Data was reported as CFU/100 mL and CFU/100 g
179 for water and oysters and the limit of detection was 1 CFU/g or 1 CFU/mL. Up to ten presumptive
180 Vp and Vv colonies per plate (when present) were stored for further species-level confirmation as
181 described below. The number reported was then multiplied by percentage of molecularly
182 confirmed (by PCR) isolates, resulting in confirmed bacterial abundance for each sample, as
183 described previously (25).

184 To test for OsHV-1 presence and abundance, the ORF100 primer set described in Burge et
185 al 2020 was used for qPCR (26). Briefly, 10 µL of PerfeCTa SYBR Green FastMix (Quantabio),
186 1 µL of each primer (ORF100 F, ORF100 R), 6 µL of water and 2 µL of DNA template were
187 mixed per reaction. All samples were run in duplicate on the 96-well Agilent ARIAMx RT-PCR
188 thermal cycler. A standard curve was generated with a synthetic plasmid of the ORF100 DNA
189 sequence by serial dilution from 30 million copies down to 3 copies.
190
191 *Statistical Analysis of Environmental and Target Bacteria Variables*
192 To determine whether the variable Site had a significant effect on concentrations of target
193 bacteria and environmental conditions, non-parametric Kruskal-Wallis tests were conducted in R
194 using the stats package. Pairwise comparisons were not conducted due to low sample number. To
195 determine whether significant correlations existed between water and oyster samples for target
196 bacteria, we conducted linear regression analyses. When data was not normally distributed as
197 determined by the Shapiro-Wilk normality test, which occurred with Vp and Vv, a Tobit
198 Regression analysis was conducted to determine significance of the observed correlation.
199
200 *DNA extraction, amplicon library preparation, and sequencing*
201 DNA was extracted from water samples using the Qiagen PowerSoil kit, and from oyster
202 tissues using the Qiagen DNEasy blood and tissue kit (Qiagen, Hilden, Germany). Digestive gland
203 and gill composites, which were homogenized at the time of collection, were digested with
204 Proteinase K prior to DNA extraction. The V4-V5 region of the 16S rRNA gene was amplified
205 using 515F-926R primers (27). Libraries were assessed for quality using an Agilent 2200
206 TapeStation (Agilent, Santa Clara, CA). Since these primers also produce amplicons from 18S

rRNA, BluePippin size selection was used to enrich for ~550bp amplicons prior to sequencing (Sage Science, Beverly, MA, USA). Additionally, 3 blanks (lysis buffer only, taken through extraction protocol, 2 extracted with tissue samples and 1 run with water samples) and 2 samples of DNA from mock bacterial communities (ZymoBIOMICS Microbial Community DNA Standard, Zymo Research, Irvine CA) were prepared and sequenced, with all samples included in the same sequencing run. After library construction, samples were sequenced using the Illumina MiSeq 2x300 (kit v.3) with custom adapters and dual barcode indices at the University of California Davis Genome Center (https://genomecenter.ucdavis.edu/). Raw data sequences and metadata are publicly available on the Qiita platform (28)(Study ID 14776) and the metadata file is published on figshare (https://doi.org/10.6084/m9.figshare.21272916).

Sequence Quality filtering and Bioinformatic Analysis

The QIIME2 pipeline was used for quality control, filtering, and bioinformatic analysis (29). R packages, including Phyloseq (30), were used for additional analyses and data visualization. Demultiplexed sequences were imported as QIIME2 artifacts, and paired reads were trimmed to remove primers, merged, and assigned ASVs using dada2 (default parameters). Taxonomy was assigned to ASVs using the SILVA database (Version 138)(31). Prior to downstream analyses, mitochondrial, chloroplast, and eukaryotic sequences were removed as well as ASVs with no identified domain.

Diversity analyses were conducted in phyloseq without rarefaction, though rarefaction at a sampling depth of 2298 (determined by alpha rarefaction curves) was also compared to confirm that results were consistent between rarefied and non-rarefied datasets. Pairwise comparisons of alpha diversity were conducted in R using a Wilcoxon rank sum test, using the Holm method for

P-value adjustment. For beta diversity analyses, Robust Atchison Principal Component Analyses (RPCA) were conducted and then visualized as biplots using the QIIME2 DEICODE plugin (32), with a minimum feature count of 10 and a minimum sample count of 500. PERMANOVA tests were used to analyze pairwise differences between groups (e.g., sample type, site) using the qiime diversity beta-group-significance command.

Log fold changes in the differential abundance of key taxonomic features in digestive gland samples were conducted in R by calculating the log ratio of targeted taxa of interest to core digestive gland microbial taxa in individual samples. Microbial taxa of interest were identified using three approaches (identification via DEICODE biplots, identification via taxonomy plots, and identification of ASVs belonging to Cyanobacteria) and analyzed separately. Core microbes were identified using the qiime feature-table core-features command, which identified 10 taxa present in >85% of samples. One of these taxa, one *Serratia* ASV, was removed from core microbiome consideration as it was also found in blank samples and is not considered a marine or oyster-associated bacterial taxa. For each sample, the abundances of taxa of interest were compared against abundances of core microbial taxa to provide differential abundance values. We used the following formula:

$$ln\left(\frac{\sum_{n=1}^{10} \text{"Taxa of interest" ASV abundance}}{\sum_{n=1}^{10} \text{"Core microbiome taxa" ASV abundance}}\right)$$

Correlations between metadata variables, including alpha diversity metrics, environmental variables, and pathogen concentrations were calculated based on Spearman's rank correlations and visualized using the corrplot package in R (33). Additional correlations between these factors and the relative abundance of taxa of interest were conducted using data from weeks 0, 1, 2, 4 for water

samples, and weeks 1, 2, and 4 from oyster samples since there was no week 0 for oysters at the designated sites based on the experimental design. Samples collected at the Kerchoff facility following depuration were not included in the correlation analyses.

Results

Environmental and oyster collection conditions at experimental sites

Experimental sites in Newport Bay, CA differed in environmental conditions including chlorophyll *a,* temperature, and salinity (Figure 1A-D, Additional File 2). Chlorophyll *a* concentration ranged from 0.72 µg/L to 38.34 µg/L (Figure 1A) and was significantly affected by site (Kruskal-Wallis rank sum test: chi-squared = 23.164, p-value = 0.017). In water samples, this metric is a common proxy for biomass of phytoplankton, which produce this photosynthetic pigment. Sites close to the back of the bay (i.e., NBS12 and NBS13) exhibited the highest observed concentrations, which occurred during weeks 2 and 3 (Figure 1A, Additional File 2). Temperature and salinity were also significantly affected by site (Temperature: chi-squared = 29.261, p-value = 0.002, Salinity: chi-squared = 23.598, p-value = 0.015). Salinity ranged from 8.4-34 ppt (Figure 1B) and temperature ranged from 20.4-26.8°C (67.7-80.2°F) across all sites (Figure 1C). Sites NBS12 and NBS13 exhibited warmer temperatures and lower salinities than other sites (Figure 1B,1C).

Target bacteria species concentrations in seawater and oysters

Fecal indicator bacteria (FIB) concentrations, which we quantified as a proxy for human fecal contamination, varied by site, and different abundance patterns were observed between water and homogenized whole oyster samples ("oyster samples")(Figure 2, Additional File 3).

Concentrations of fecal coliform bacteria (FC), *Escherichia coli* (EC), and *Enterococcus* spp. (ENT) in water samples were all elevated at sites 12 and 13 compared to samples collected at other sites during the same week (Figure 2) and were overall significantly affected by site (FC: chi-squared = 23.476, p-value = 0.015, EC: chi-squared = 28.76, p-value = 0.002). FC and EC concentrations in water also appeared to be elevated at site 6, though positive samples were observed at all sites during the experiment. In oyster samples, FC and EC concentrations were elevated at site 7 compared to the other sites (Figure 2, Additional File 3) and were also significantly affected by site (FC: chi-squared = 23.921, p-value = 0.013, EC: chi-squared = 25.418, p-value = 0.008). Oyster and water concentrations were not correlated for *E. coli* and fecal coliforms (Linear Regression, E. coli: F-statistic: 0.6927, adjusted p-value: 0.411, fecal coliform: F-statistic: 1, adjusted p-value: 0.1916) (Additional File 3). Water column concentrations of FC and EC exceeded the California SHEL standard of 43 MPN/100 mL at 3 sites: NBS6, NBS12, and NBS13.

Vibrio species of concern for human health also varied in concentration by site but unlike with FIB, oyster and water samples were positively correlated, particularly for *Vibrio parahaemolyticus* (Vp) (Figure 2, Additional File 3). In water, *Vibrio* spp. targets were highest at sites 12 and 13 compared to different sites measured during the same week (Figure 2), which generally corresponded to warmer temperatures and lower salinities (Figure 1B,1C). Maximum Vp concentrations exceeded 30,000 CFU/100 mL (Site NBS13, Week 2, Additional File 2) while highest *V. vulnificus* (Vv) concentrations were >100,000 CFU/100 mL (Site NBS12, Week 2). These species were also observed at most other sites during the experiment, albeit in lower concentrations. Site had a significant effect on water concentrations of both Vp and Vv (Vp: chi-squared = 29.351, p-value = 0.002, Vv: chi-squared = 22.371, p-value = 0.022). However, Site did

297 not significantly affect Vp or Vv concentrations in oysters (Vp: chi-squared = 18.715, p-value =
298 0.066, Vv: chi-squared = 9.5502, p-value = 0.571). In oyster samples, Vp concentrations were also
299 highest at sites 12 and 13 compared to other sites, except for during week 1 when concentrations
300 were also elevated at site 11. Vv was only detected in 3 oyster samples, and the highest
301 concentration (177,500 CFU/100 g oyster tissue) was observed during week 1 at site NBS13.
302 Correlations between water and oyster concentrations were positive and significant for *V.*
303 *parahaemolyticus* (Tobit Regression, Wald-statistic: 5.862, p-value: 0.015) and for *V. vulnificus*
304 (Tobit Regression, Wald-statistic: 5.917, p-value: 0.015), though for V. vulnificus only 3 datapoint
305 had positive values for both oyster and water concentrations, thus further validation is needed
306 (Additional File 3).

307 In addition to bacterial targets, we used qPCR to screen for the presence of *Ostreid*
308 *herpesvirus 1* (OsHV-1) in whole oyster samples, which is a virus that commonly infects Pacific
309 oysters causing mass mortality events. It has previously been detected in adult Pacific oysters in
310 Southern California (29). We did not detect the virus in any of our samples.

311

312 *Influence of sample type on microbial community diversity and composition*

313 We characterized microbial communities using 16S rRNA V4-5 amplicon sequencing for
314 4 different sample types: water, whole oysters, oyster gill tissue, and oyster digestive glands (DG).
315 After filtering for quality, ~11M sequences were generated across samples and controls,
316 representing ~33K unique amplicon sequence variants (ASVs) belonging predominantly to
317 bacteria.

318 Sample type was a major driver of microbial diversity, with significant differences in both
319 alpha and beta diversity. Water samples had higher species richness (Observed ASVs) than all

oyster sample types, and water and whole oysters had higher alpha diversity (Shannon Diversity) than the oyster digestive gland and gill tissue (Figure 3A and 3B, Additional File 4). Sample type significantly influenced beta diversity (PERMANOVA, pseudo-F: 122.014, p-value: 0.001, Additional File 4), and sample types significantly differed from each other based on pairwise PERMANOVA tests (RPCA analysis, Figure 3C, Additional File 4).

Specific microbial taxa were strongly associated with either oyster or water sample types, or in some cases, specific oyster tissues. Key microbial features characteristic of oyster samples included members of the *Mycoplasmataceae* (Class Bacilli) and *Spirochaetaceae* families (Class Spirochaetia)(Figure 3C), which were also among the most relatively abundant taxa found in oyster tissues generally (Additional File 5, Additional File 6). Key water taxa included marine members of SAR11 *Clade Ia*, *HIMB11*, and *Planktomarina* (Figure 3C), which are all Alphaproteobacteria. While oyster gill and DG microbiomes shared some common microbial taxa, others were more representative of either sample type. For example, members of the genus *Blastopirellula* (Class Planctomycetes) and genus *Spiroplasma* (Class Bacilli) were more representative of DG samples, while *Sphingoaurantiacus* (Class Alphaproteobacteria), and Spirochaetia ASVs had high relative abundance in gill but not DG samples. (Additional File 6).

Influence of site and environment on microbial community diversity and composition

Water microbial community diversity was impacted by site and environmental conditions to a greater extent than oyster microbiomes, which were comparably stable across sites. Beta diversity of water samples differed at back bay sites, which also exhibited differences in temperature, salinity, and chlorophyll *a* concentration (Figure 1). Specifically, microbial communities from NBS12 and NBS13 were significantly different from all other sites, and NBS11

was significantly different from all sites except 9 and 13 (Figure 3C, Additional Files 4 and 7). Communities from NBS12 and NBS13 were distinct from other sites on the biplot, as indicated by the grey dashed box surrounding these samples (Figure 3C), and in pairwise distance comparisons between sites within water samples (Additional File 7). Alpha diversity (Observed ASVs and Shannon Diversity) was negatively associated with temperature and positively associated with salinity in water samples, suggesting lower diversity at bay back sites (Additional File 8).

Beta diversity in oyster samples did not significantly differ across sites and environmental conditions, but there were site-specific differences in taxonomic composition and environmentally linked differences in tissue-specific alpha diversity. Whole oyster and DG samples had no statistically significant differences in alpha diversity across the variables tested; however, gill alpha diversity was associated with warmer temperatures (Additional File 8). Water and DG samples exhibited high relative abundance of one cyanobacterial ASV, which dominated microbial communities at Site 12 during weeks 2 and 4 (Additional File 5). The closest taxonomic assignment for this ASV was *Cyanobium* PCC-6307, which is commonly found in freshwater aquatic environments. Chlorophyll *a* levels were high during these sampling points which may suggest high actual and not just relative abundance of these cyanobacteria.

Additional differences in oyster microbial community taxonomy were observed at NBS12. Key taxa associated with DG microbiomes (Figure 4A, taxa annotated with arrows) had lower relative abundance at this site compared to the core DG microbiome (Figure 4B). Additionally, during week 2 in DG samples, a Chlamydiales ASV comprised ~30% of the microbial community. These are often intracellular animal pathogens and have been hypothesized to be linked to Oyster Oedema Disease (OOD) in pearl oysters (30). Both the *Cyanobium* and Chlamydiales ASVs had higher differential abundance in relation to the core DG microbiome at NBS12 compared to other

366 sites (Figure 4C, 4D). The increase of these two ASVs at this time point also manifested as a
367 community with distinct beta diversity (this data point is enclosed in a gray dotted box in the biplot
368 in Figure 4C), however, this could not be statistically tested as oysters in this sample were pooled
369 as a single data point.
370
371 *Relationship between potential bacterial pathogens and water or oyster microbial communities*
372 We investigated whether certain characteristics of water and oyster microbial communities,
373 namely community diversity and prominent microbial taxa in these sample types, were associated
374 with high or low concentrations of fecal indicators and target *Vibrio* species.
375 In water samples, microbial communities with higher fecal indicator and *Vibrio* spp. levels
376 were typically found at the back bay sites NBS12 and NBS13 and were significantly different in
377 beta diversity compared to all other sites (Additional File 4, Additional File 7). Target bacteria
378 concentrations coincided with lower salinity, warmer temperatures, and lower alpha diversity
379 (Figure 1, Figure 3D). Both fecal indicator bacteria and potentially pathogenic *Vibrio* spp. were
380 found in lower concentrations near the front bay sites and were significantly associated with higher
381 salinities and linked to ASVs common to marine environments (e.g., SAR11 *Clade Ia*, *HIMB11*,
382 and *Planktomarina*)(Figure 2, Figure 3, Additional File 9). Some specific microbial taxa were
383 linked to low salinity conditions found at back bay sites and high potential pathogen concentrations
384 (Additional File 9). For example, a *Flavobacterium* ASV was positively associated with multiple
385 bacteria of concern, including elevated *Vibrio parahaemolyticus* (Vp) concentrations in both water
386 and oysters. Additionally, *Cyanobium* PCC-6307 was abundant at sites and times when relatively
387 high Vp levels were also observed (Site NBS12, weeks 2 and 4).

We also investigated whether the relatively high target bacteria concentrations observed in the water at sites NBS12 and NBS13, in environmental conditions that were potentially stressful for oysters, were also linked to changes in oyster microbial communities. As opposed to water samples, fecal indicator and *Vibrio* spp. concentrations in oyster samples didn't exhibit clear trends except in the case of Vp, which was highest at back bay sites in both water and oyster samples (Figure 2). Similarly, beta diversity within oyster sample types did not vary significantly with site when grouped across weeks, though we did observe that the oyster DG microbial community where the highest concentration of Vp was observed (Week 2, Site NBS12) visibly separated from most DG samples in the DEICODE biplot (Figure 3C, gray dotted box).

Several oyster-associated microbial taxa were associated with either fecal indicator or *Vibrio* spp. concentrations in oysters. In whole oyster samples, *Mycoplasma* and *Blastopirellula* ASVs were negatively correlated with concentrations bacterial targets in water (Additional File 10) . We also separately assessed gill and digestive gland (DG) microbiomes. In DG microbiomes, most of the high relative abundance taxa were not linked to environmental conditions or concentrations of human-health associated bacteria (Additional File 11). However, ASVs belonging to the *Blastopirellula* and *Mycoplasma* genera were negatively associated with *V. parahaemolyticus* in oysters, which likely drove the trend observed in whole oysters. Additionally, *Fuertia* bacteria (Order: Planktomycetales) were associated with lower levels of *E. coli* and Fecal Indicator bacteria. In Gill samples, most of the dominant taxa besides *Mycoplasma* ASVs were not significantly associated with environmental variables or human health-associated bacteria (Additional File 12). The cyanobacteria taxa *Cyanobium* PCC-6307 dominated water and oyster DG microbial communities where the highest concentrations of Vp were observed in oysters (Site NBS12, weeks 2 and 4), and where environmental conditions varied from other sites (Figure 1).

411 In oyster DG samples, *Cyanobium* PCC-6307 was positively associated with Vp concentrations in
412 oysters, and positively associated with high concentrations of both the fecal indicators and *Vibrio*
413 spp. quantified in water samples, meaning that when *Cyanobium* PCC-6307 was abundant in oyster
414 DG microbiomes, potentially pathogenic bacteria concentrations were relatively high in the water
415 and Vp was relatively high in the oysters (Additional File 11).

416 In addition to examining correlations between pathogen concentrations and relative
417 abundance of microbial taxa in these sample types, we also used a differential abundance approach
418 to assess whether other microbial taxa that were common in other DG or water samples decreased
419 in relation to other taxa (i.e., a compositionally aware approach rather than solely examining
420 relative abundance). This addressed the issue of whether the observed decrease in relative
421 abundance of key taxa was due solely to the proportional increase in *Cyanobium* PCC-6307, or
422 whether ratios of these taxa to the core DG microbiome increased as well. While the log ratio of
423 *Cyanobium* to core DG taxa increased at site NBS12, the ratio of several key taxa of interest (as
424 indicated by the DEICODE RPCA analysis and biplot, Figure 4A) decreased in relative abundance,
425 suggesting that there was a decrease in the abundance of these taxa.

426 In investigating these associations between oyster microbiomes and fecal indicator and
427 *Vibrio* spp. concentrations, some possible confounding variables in this dataset include the
428 possibility that oyster microbiomes may change over time following translocation and that
429 pathogens may take time to accumulate in oyster tissues. Specific properties of the sites where
430 oysters were collected could influence oyster microbiomes after pooling, depuration, and
431 deployment, and it is unknown how rapidly the oyster microbiomes stabilize and adapt to new
432 conditions. Additionally, pooling multiple oyster samples, which is a common methodology for
433 quantifying human pathogens, obscures variability of individual oysters in samples. Furthermore,

434 the sites included in this study had varied physicochemical profiles. Although this allowed us to
435 explore the effects of salinity and temperature, additional factors that were not measured may differ
436 between sites and could influence the results as well. These present some limitations to the current
437 experiment that should be considered.
438
439 **Discussion**
440 *Oyster and water microbiota are differentially influenced by the environment*
441 To investigate links between microbiomes and pathogen accumulation in bivalves, we first
442 analyzed the microbial communities of water and examined their respective environmental
443 influences. Though Pacific oysters originated in Japan, they are now extremely common along the
444 North American West coast. However, the microbial communities and host interactions of these
445 successful colonizers are poorly understood. Prior studies of regional Pacific oyster microbiomes
446 observed similar microbial communities between extrapallial fluid, mantle fluid, and water
447 microbial communities (34) and characterized impacts of different diets on fecal microbiomes
448 (35). Our study focused on whole oyster as well as gill and digestive gland (DG) microbial
449 communities, which are important to host physiology and may be prone to concentrating
450 environmental bacteria since gills filter water particles and the DG may receive bacteria attached
451 to food particles. All sample types we examined had significantly distinct microbial communities
452 (Figure 3C, Additional File 5), which is consistent with prior studies showing that microbiomes of
453 many animals, including oysters have tissue-specific microbial communities (36, 37).
454 Understanding how the environment influences oyster microbiomes is critical to preserving
455 their ecosystem functions and supporting safe and abundant shellfish harvesting. We observed key
456 taxonomic features among oyster sample types that may play a role in maintaining host health or

457 reflect environmental adaptations. The dominant tissue-specific taxa we observed are common in
458 Pacific oysters from diverse geographic regions, suggesting adaptation to oyster
459 microenvironments and potentially host interactions. Furthermore, the tissue-specific communities
460 were similar to each other across sites despite differing significantly from the surrounding water
461 microbiomes. In particular, Spirochaetaceae and Mycoplasmataceae taxa were common in all three
462 oyster sample types, with some ASVs more represented in specific sample types (Additional File
463 6).

464 Spirochaetaceae taxa were observed in *C. gigas* gill samples from the Wadden Sea,
465 Tasmania and New South Wales (NSW), Australia (36–39). Members of this can cause human
466 disease (e.g., Lyme disease and syphilis), but are also commonly associated with marine animals
467 ranging from gastropods to corals, and provide beneficial host functions including nitrogen and
468 carbon fixation (40–43). Mycoplasmataceae bacteria were common in DG samples, a pattern
469 observed in above studies and in samples from France (44) and in Olympia oysters (*Ostrea lurida*)
470 from the Northwest US (45). Interestingly, this was not a dominant DG microbial group in Pacific
471 oyster DG samples from Mexico, however, this may be due to different analytical methods (46).
472 Like Spirochaetaceae, several Mycoplasmataceae taxa are implicated in human and animal disease
473 while other related taxa naturally reside in animal microbiomes with potentially symbiotic roles.
474 A metagenomic study of Eastern oyster (*Crassostrea virginicus*) microbiomes identified a
475 dominant *Mycoplasma* sp., and genomic and metabolomic reconstructions revealed a reduced set
476 of metabolic functions and high reliance on host-derived nutrients (47). A reanalysis of previous
477 *C. virginicus* from publicly available studies suggested that members of the parent Mollicutes class
478 were highly prevalent in adult oyster DGs and lower in larva and biodeposits. For these taxonomic
479 groups, their role in oysters or shellfish generally has not been characterized but may be of wide-

480 spread importance, thus changes in the relative abundance of these taxa due to environmental or
481 other stressors could have negative host impacts.

482 Community diversity of water strongly reflected the collection sites and associated
483 environmental conditions, while in oyster samples beta diversity was more stable among sites and
484 environments, but the relative abundance of key microbial taxa and alpha diversity differed.
485 Variations in water microbial communities likely reflect diverse and dynamic coastal processes.
486 Oysters, like many other animals, can to some extent regulate their microbial communities, though
487 under stressful conditions these communities may shift from a stable to an unstable state (18, 48,
488 49). As our experimental sites represent a range of environmental conditions, particularly distinct
489 at sites NBS12 and NBS13, we predicted that microbial communities would differ across sites.
490 The lack of beta diversity divergence in oyster microbial communities deployed to sites with
491 varying physicochemical water parameters and distinct water microbiomes (Figure 3C, Additional
492 File 7) may reflect a strong selection pressure (host and/or microbe-mediated) for the structure of
493 these communities, though it is also possible that short-term (i.e., weeks) exposure to these
494 conditions does not impact community diversity, but that long-term exposure would. Alpha
495 diversity was higher in gill (Observed ASVs and Shannon Diversity) and DG (Shannon Diversity)
496 samples (Additional File 8) at the environmental conditions present at sites NBS12 and NBS13
497 (low salinity, high temperature), however, we did observe a negative association between these
498 conditions and key microbial taxa present in DG tissues (Additional File 8), particularly members
499 of the Mycoplasmataceae family discussed above, which could reflect a detrimental impact to
500 hosts if these bacteria are beneficial.
501

Potential human pathogen concentrations were linked to specific environments and microbial community features

Terrestrial and marine human pathogens often have distinct environmental abundance patterns based on their natural environments, but for both classes understanding associations with the marine environment and co-occurring microbes in water and oyster tissues may help characterize and ultimately predict bivalve pathogen concentrations. Some factors that likely play a role in bivalve pathogen accumulation include environmental pathogen concentrations, host behavior (e.g., feeding), pathogen interactions with bivalve transmission vectors, host physiology (including microbiome states), and microbial features that enable host colonization. Our study investigates these latter microbial variables, which are a key and understudied factor in human pathogen ecology in the marine environment.

Animal microbiomes are often linked to organismal health and the environment, and stressful environmental conditions can cause increases in the occurrence and relative abundances of otherwise uncommon bacterial taxa. In edible bivalve species, this may include an increase in human pathogens, but this possibility has not been adequately investigated. We predicted that oysters translocated to potentially stressful locations (i.e., sites NBS12 and NBS13) would concentrate more human pathogens, with coinciding microbial community changes. This occurred in the case of *Vibrio parahaemolyticus* (Vp), the most common cause of seafood transmitted vibriosis disease (4) but did not occur with regards to *V. vulnificus* (Vv) or fecal indicators measured, which exhibited variable patterns across environmental sites and over time. In water, FIB and *Vibrio* spp. bacteria were most abundant at sites NBS 12 and NBS13, consistent with their affinities for low salinity and warm temperatures. Fecal indicator and *Vibrio* spp. concentrations in oysters, however, were generally not linked to water concentrations or environmental conditions

525 (except for Vp, discussed below), though in the case of Vv the low number of detections could
526 confound the correlation analysis. It is possible that oysters with stable microbial communities
527 may accumulate environmental human pathogens through feeding/filtering but possess
528 microbiome-regulated controls on total accumulation. This would explain why, with the exception
529 of Vp, oysters in water that contain relatively high Vv or FIB concentrations (i.e., the back bay
530 sites NBS12 and NBS13) don't accumulate proportionally more of these organisms than oysters
531 at "low pathogen" sites. This also confirms that while environmental conditions conducive to
532 pathogen proliferation may be necessary for high concentrations in water, they are not sufficient
533 for accumulation in shellfish.

534 In the case of Vp, concentrations in water and oyster samples were positively correlated
535 and linked to changes in oyster microbial communities, particularly at site NBS12, and to relatively
536 warm temperature, low salinity environmental conditions. Prior studies have observed stress-
537 induced increases and changes in *Vibrio* spp. relative abundance in oyster microbiota, but these
538 studies typically focus on *Vibrio* spp. that are not human pathogens and typically do not quantify
539 the bacteria (36, 50). The highest oyster Vp concentrations were observed at NBS12 during week
540 2, which featured a distinct DG microbial community (Figure 4C [small gray dotted box]). These
541 high Vp concentrations coincided with an increase in the relative abundance of two uncommon
542 DG taxa that could suggest an unhealthy host state: a Chlamydiae family ASV, which are often
543 intracellular animal pathogens and potentially cause Oyster Oedema Disease (OOD) in pearl
544 oysters (30), and a cyanobacteria taxon *Cyanobium* PCC-6307, which was commonly present in
545 both water and oyster DG samples (Additional File 5, Figure 4B, 4C). Meanwhile, several common
546 DG oyster taxa (e.g., *Mycoplasma* and *Blastopirellula*) were less relatively abundant at the "high
547 Vp" back bay sites. Since *Mycoplasma* bacteria are considered core members of oyster DG

548 microbial communities and have been linked to increased oyster survival (45) the increase in rare
549 taxa and decrease in core taxa may suggest poor host health and microbial community dysbiosis.
550 Despite these observations at NBS12, Vp was also consistently abundant at site 13, which had
551 similar environmental conditions but not these divergent taxa. This may suggest that both the
552 environment and specific microbial taxa in oysters and the environment act synergistically to
553 enable marine human pathogen accumulation in oysters. Other factors may also be involved,
554 including time oysters are exposed to stressful environments and oyster feeding behavior.
555
556 *Phytoplankton links to oyster pathogen accumulation*: *signs of dysbiosis or transport vectors?*
557 The highest concentration of *Vibrio parahaemolyticus* bacteria we observed in oysters,
558 which causes an estimated 45,000 cases of vibriosis annually in the US (4), was positively linked
559 to elevated relative abundance of a cyanobacteria ASV in both water and oyster DG samples. This
560 led us to consider whether the abundance of this taxon signaled dysbiosis in oyster DG
561 microbiomes, whether cyanobacteria were acting as a transport vector for oyster pathogen
562 accumulation (via feeding), or potentially both. Chlorophyll *a* concentration was positively
563 associated with Vp (Figure 3D, 3E) and was also highest at NBS12 compared to samples collected
564 the same week at other sites, which coincided with the observed cyanobacterial taxa (Additional
565 File 6). This supports the possibility that these cyanobacteria were abundant in the microbial
566 community, which would increase their likelihood of interacting with Vp, though we did not
567 measure actual attachment and other phytoplankton taxa may produce chlorophyll *a* in the
568 environment.
569 Since oysters are filter-feeders that consume particles and phytoplankton prey, positive
570 associations between pathogens and aquatic taxa such as *Cyanobium* spp., could indicate vibrio

571 attachment mediating oyster pathogen accumulation. Vp attachment to phytoplankton could
572 enable environmental persistence by providing nutrients and attachment substrate while increasing
573 oyster accumulation if they are subsequently preyed upon, as both terrestrial and marine pathogens
574 frequently attach to marine particles and form highly concentrated biofilms. We previously
575 identified phytoplankton taxa positively linked to high Vp concentrations in southern California
576 water microbiomes, including common oyster prey species (e.g., *Thalassiosira* spp. diatoms) and
577 a cyanobacterial ASV *Prochlorothrix* sp. PCC-9006 (13), though we did not assess oyster
578 pathogen concentrations in that study.

579 It is unclear whether Vp actually attaches to cyanobacteria in the environment, and whether
580 cyanobacteria could serve as a vector for Vp uptake by oysters. Vp interactions have been
581 demonstrated in a laboratory setting for cyanobacteria and other phytoplankton taxa (15, 51), and
582 attachment would be consistent with the widespread particle-attaching lifestyle of *Vibrio* species.
583 While cyanobacteria are often small and not known to be a major bivalve food source, *Cyanobium*
584 spp. can form larger cell aggregates (52), which would enhance their potential for both vibrio
585 attachment and oyster uptake. Given the other changes observed in DG microbiomes that
586 coincided with high Vp abundance, cyanobacteria abundance may also represent transient
587 environmental bacteria accumulating in oysters due to altered host microbiome states and
588 potentially impaired host health. Our observation that alpha diversity was higher in DG samples
589 at these Back Bay sites may suggest hosts were not actually impaired since lower alpha diversity
590 is often linked to impaired host states. However, recent studies have called this paradigm into
591 question with observations that alpha diversity is not necessarily linked to host health (53, 54). A
592 recent study of Eastern oysters observed no significant differences in alpha diversity between
593 infected and uninfected individuals for most tissue types, including gut microbial communities

(47). Furthermore, microbiome diversity is far from the sole indicator of host stress. While we are not able to discern from our study which of these scenarios occurred, or if it was a combination of factors, this represents an important hypothesis to test in future studies.

Several future research directions can further strengthen our understanding of human pathogen ecology in the marine environment and enable safe seafood consumption now and in future ocean conditions. Investigating changes in the eukaryotic microbiota may reveal additional vibrio transmission vectors and microbiome dynamics linked to host health not clear from bacterial microbiota analyses. Integrating knowledge of the oyster microbiome with host physiology, particularly feeding behavior, will help elucidate the interactions between both host and microbe-mediated drivers of bivalve pathogen accumulation, particularly in relation to predicted future ocean changes. This could also include assays to observe the actual attachment of pathogenic *Vibrio* spp. to environmental microbes and oyster tissues (e.g., FISH (55). Furthermore, better understanding the microbial taxa either positively or negatively linked to oyster pathogen accumulation is essential to characterizing the ecology of these infectious disease organisms and could ultimately lead to developing bioindicators of oyster pathogen accumulation or identifying taxa that could be used as defensive probiotics.

Conclusion

Understanding factors beyond environmental conditions that drive human pathogen accumulation in bivalves is critical to supporting safe and sustainable seafood consumption. Here we demonstrate that both environmental factors and microbial communities interact to differentially influence concentrations of potential human pathogens in water and oysters. While specific environmental conditions are linked to both microbial community diversity and

617 concentrations of potential pathogenic *Vibrio* spp. and indicator species indicative of fecal
618 contamination in water samples, concentrations of these bacteria in water are not necessarily linked
619 to concentrations in oysters. Oyster microbiomes and pathogen concentrations were less
620 environmentally dependent than those in water, except in the case of *Vibrio parahaemolyticus*,
621 where relatively high oyster concentrations were associated with an increase in environmental
622 cyanobacteria and a decrease in the relative abundance of key digestive gland taxa. This study
623 suggests that environmental conditions and microbial communities may interact, potentially
624 synergistically, to drive human pathogen concentrations in oysters. Future research integrating
625 pathogen attachment to oyster uptake vectors in the environment, oyster behavior and physiology,
626 and the functional roles of oyster microbiomes and specific taxa - particularly in response to
627 changing environmental conditions - will provide data critical for promoting safe seafood
628 harvesting for a growing human population in the future.
629

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638
639
640

641 **Author Contributions**

642 R.E.D., A.Z-F., E.C., J.G., and A.E.A. were responsible for the conception and design of the study. R.E.D.,
643 A.Z-F., and E.C. were responsible for collecting and processing samples. R.E.D., A.Z-F., S.A., S.M.K.,
644 E.K., and Y.G. conducted data analysis. R.E.D., A.Z-F., S.A., and J.G. contributed to data
645 interpretation and manuscript preparation.

646

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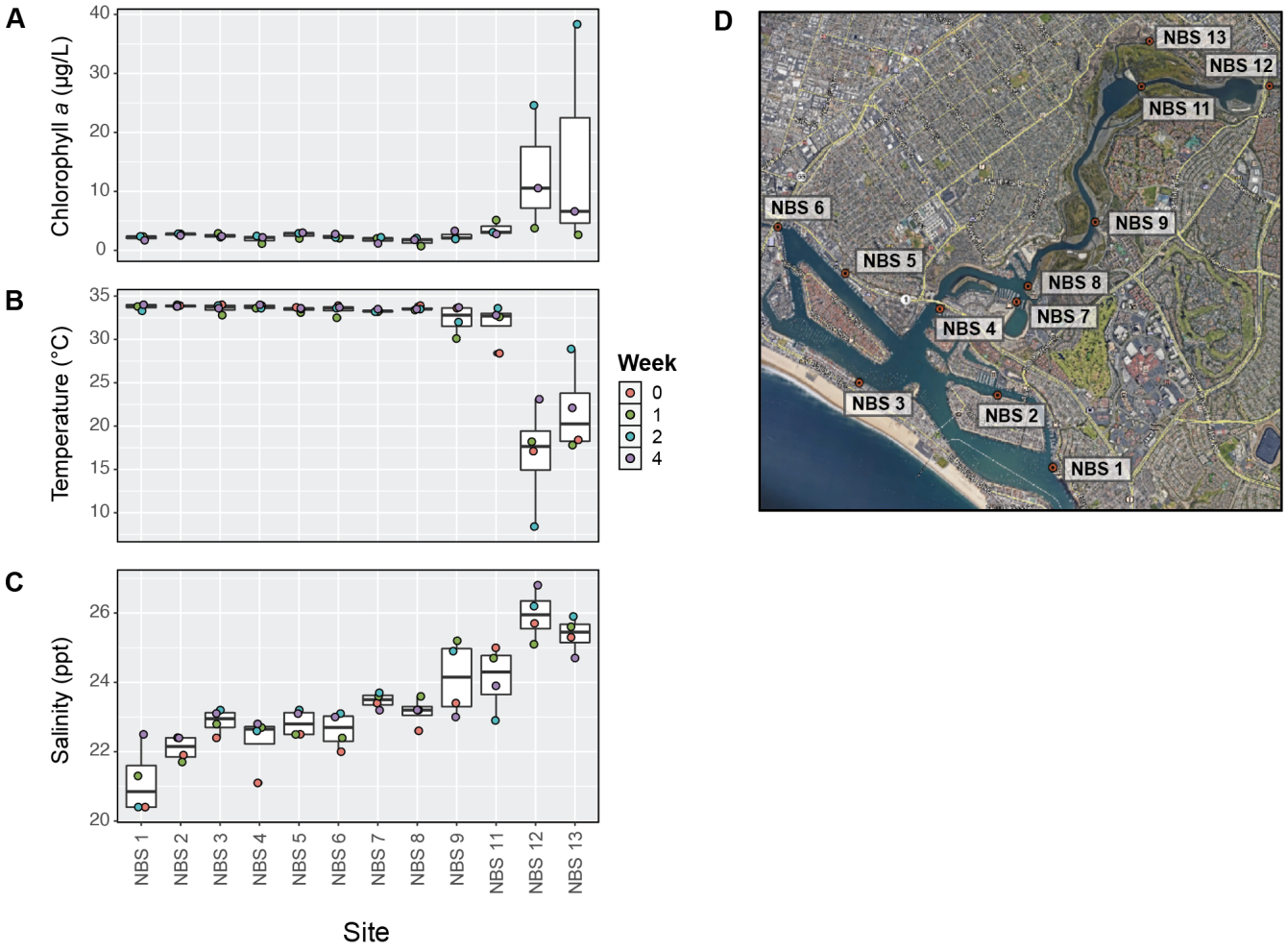


Figure 1: Experimental sites and environmental conditions. Environmental conditions at the time of sample collection, including (A) chlorophyll *a* concentration, (B) temperature, and (C) salinity. (D) Sites in Newport Bay, CA where oysters were deployed and water and oyster samples were collected for the study.

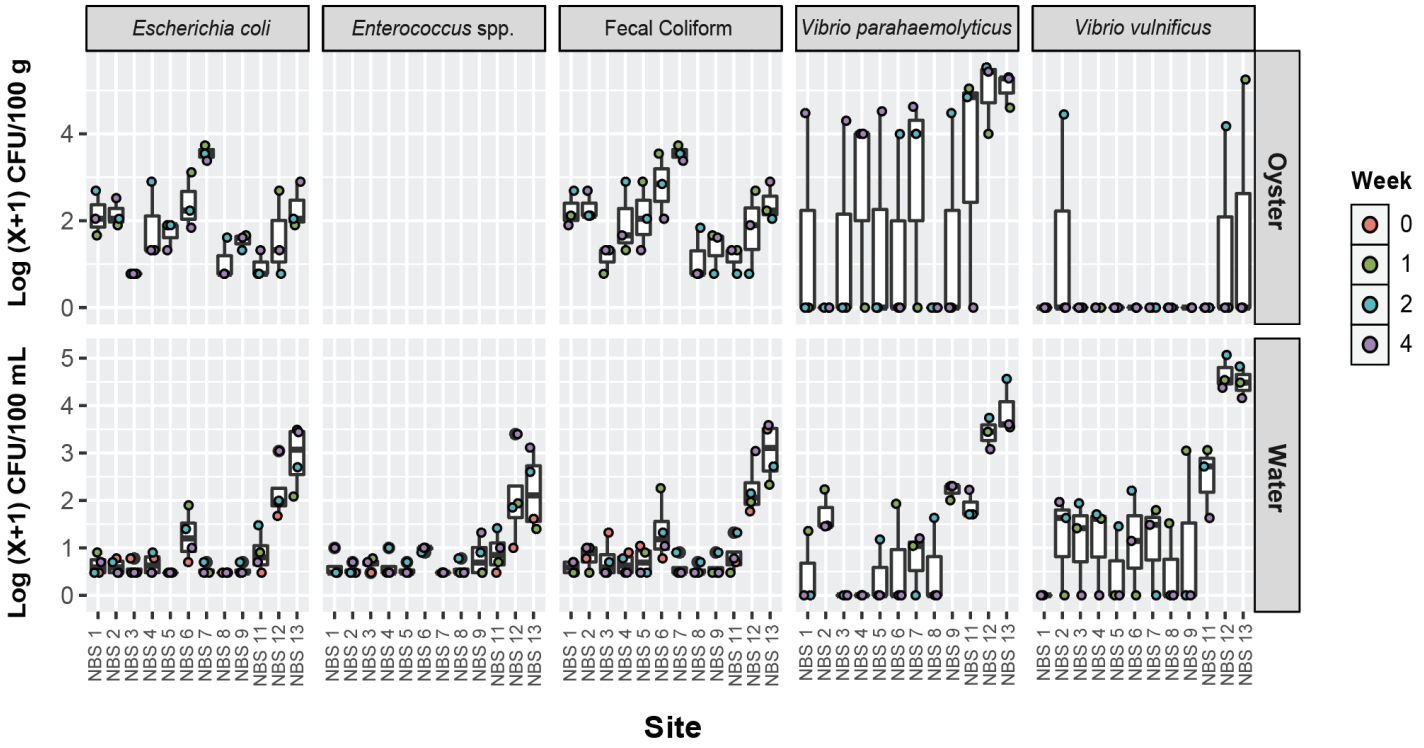
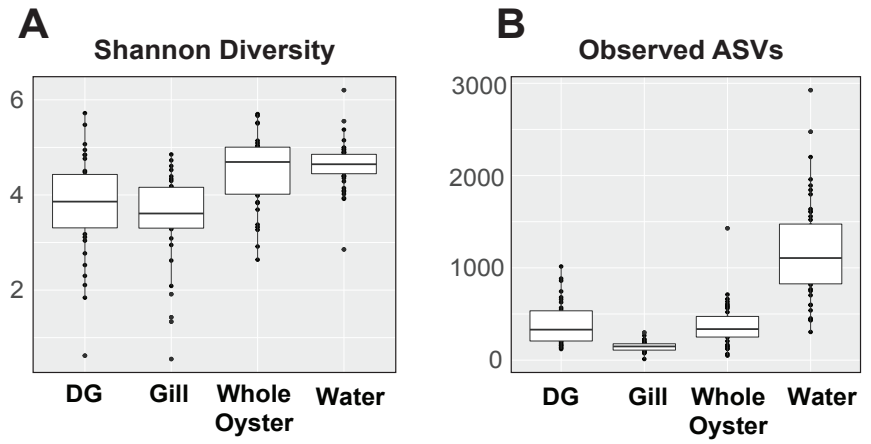


Figure 2: Fecal indicator bacteria (FIB) and pathogenic *Vibrio* spp. concentrations in oysters and water. Concentrations are shown as Log (x+1) CFU/100g of oyster tissue or 100 mL of seawater. FIB concentrations in oysters were calculated using the MPN method.



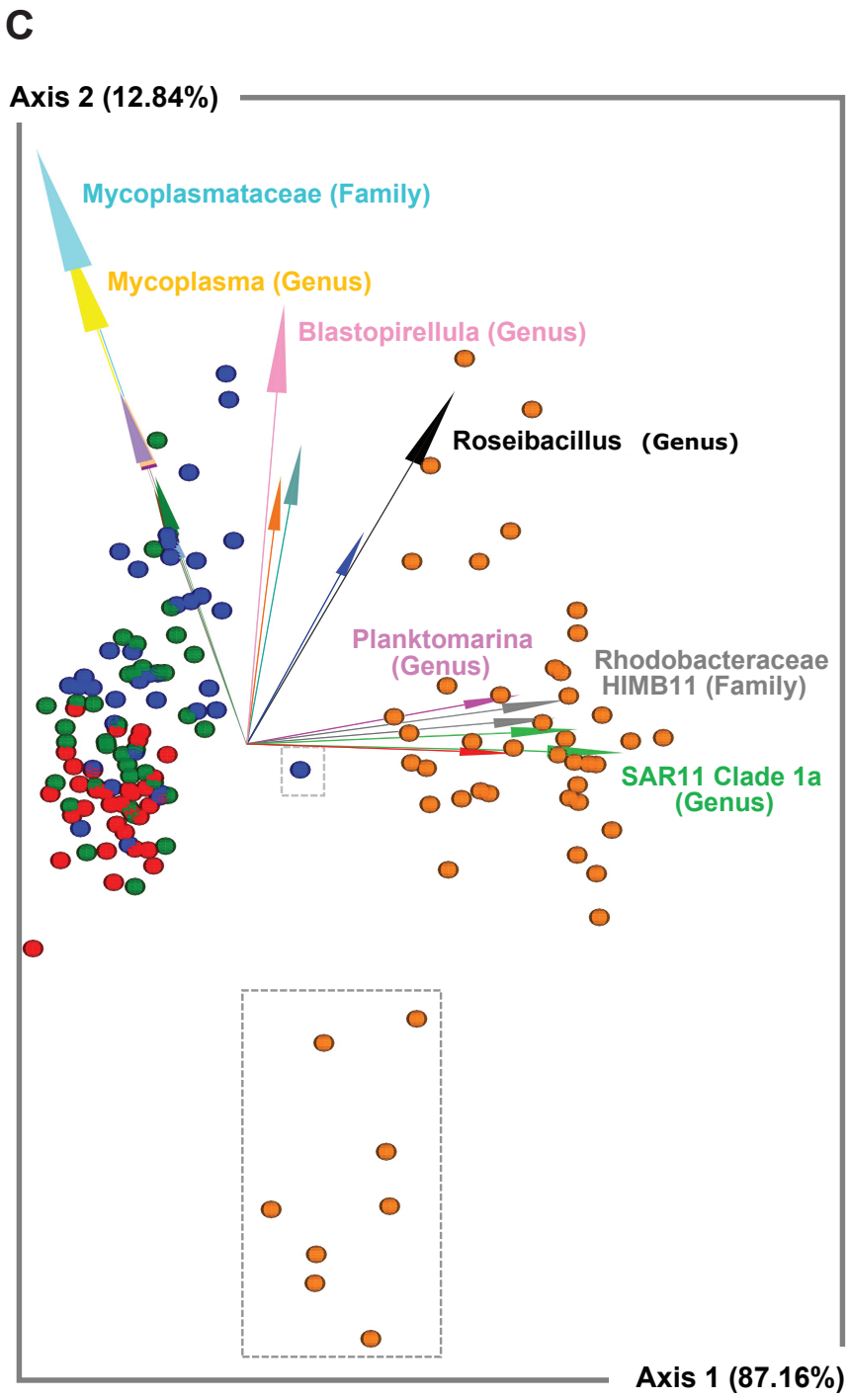


Figure 3: Diversity of water and oyster microbial communities and relationships with environmental parameters and pathogen concentrations. (A) Shannon diversity and (B) Observed ASVs compared between sample types. (C) DEICODE biplot showing RPCA distance between samples colored by sample type (blue = digestive gland, green = whole oyster, red = gill, and orange = water) and arrows depicting the key microbial taxa driving diversity, with annotation to the highest available taxonomic annotation noted. Water samples from sites NBS12 and NBS13 are enclosed in the larger gray dotted box, and a DG sample from NBS12 at time point 2 is enclosed in the smaller gray box.

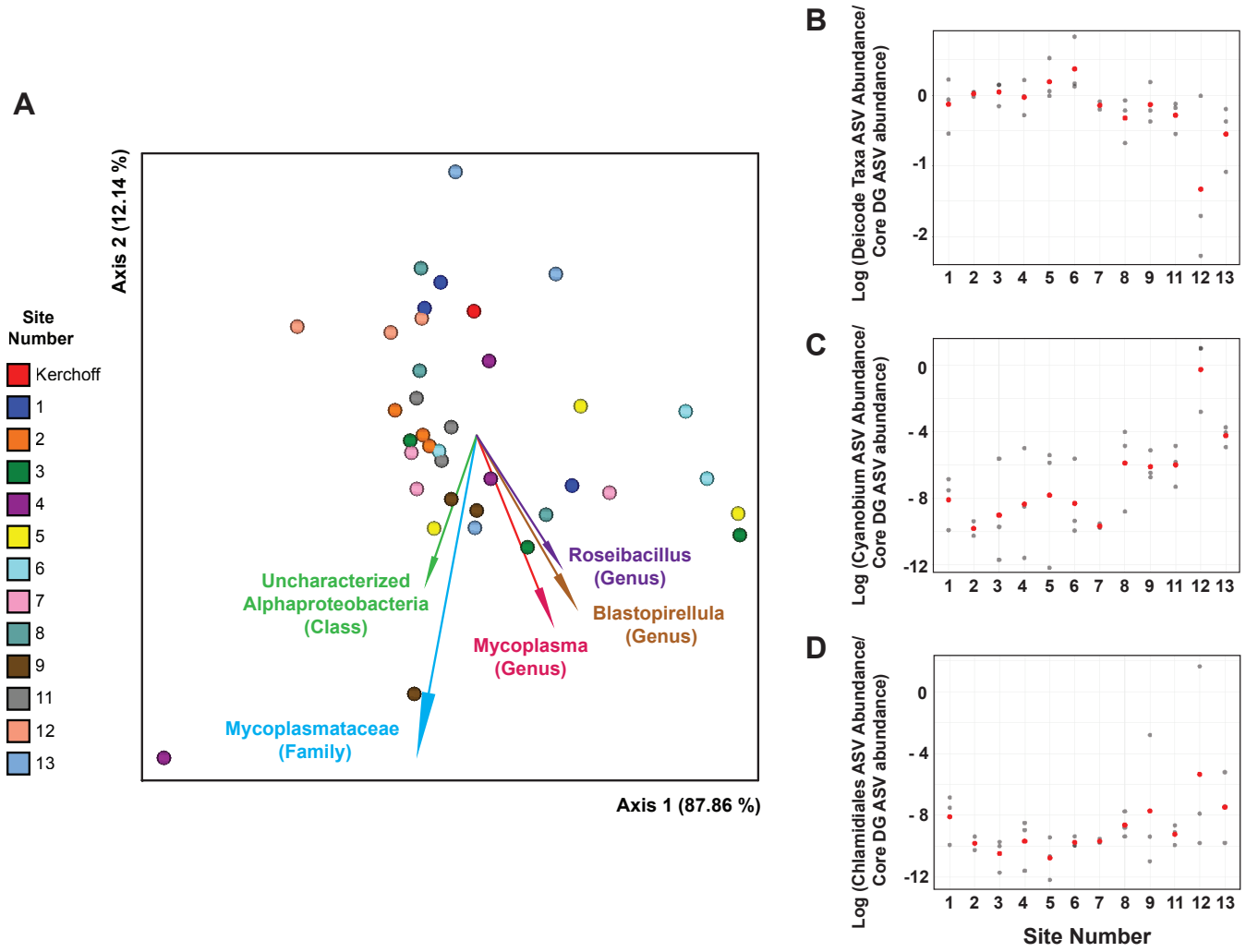


Figure 4: Differentially abundant taxa across sites in oyster digestive gland tissues. (A) RPCA biplot of digestive gland samples with markers depicting individual samples and marker colors indicating site (shown in legend). Arrows depict microbial taxonomic features driving differences in community composition (beta diversity) with the best available taxonomic composition. The log fold-change in differential abundance (compared as a ratio to the core digestive gland microbiome) is shown for (B) the driving taxonomic features from the RPCA DEICODE analysis (taxa with arrows), and two specific ASVs common at back bay sites: (C) Cyanobium PCC-6307, and (D) a Chlamydiales ASV. Red markers denote the average log-fold change.