COMMUNITY ECOLOGY AND DISEASE DYNAMICS IN PACIFIC OYSTERS: UNRAVELING MICROBIOME-PATHOGEN INTERACTIONS IN THE WILD

By

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ABSTRACT

COMMUNITY ECOLOGY AND DISEASE DYNAMICS IN PACIFIC OYSTERS: UNRAVELING MICROBIOME-PATHOGEN INTERACTIONS IN THE WILD

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In the context of multispecies microbial assemblages, disruptions can occur when there are alterations in host conditions, such as the onset of a disease. Notably, viruses have the potential to reshape a host's microbial community. However, the role of the host's habitat and environment, which could be pivotal in communities with shifting niche space and habitat filters, is often overlooked in host-microbe-pathogen interactions. Recognizing the importance of these factors, I employed a field-based approach to understand microbial community dynamics in the presence of disease. To address the influence of geographical location, I conducted an analysis involving healthy and infected oysters at two distinct sites (Tomales Bay, CA and Humboldt Bay, CA) using hypotheses based upon previous laboratory research on Ostreid herpesvirus (OsHV-1) effects on oyster microbial community. Using 16S rRNA sequencing data and qPCR data, I shed light on the significant impacts of host location and habitat in disease systems, emphasizing their importance in disease research. I found that, 1) microbial community dynamics were impacted by OsHV-1 and geographical location of sample collection. However, the presence of OsHV-1 did not result in decreased richness, diversity, or evenness in the microbial community, contrary to previous research. 2) OsHV-1 infection

did not inhibit the oyster microbial community's ability to filter its environment. 3) Contrary to expectations, *Vibrio* abundance did not exhibit a significant increase with OsHV-1 load. 4) The microbial community in infected samples did not exhibit dominance by *Vibrio*, again, contrary to previous laboratory-based results. These findings shed light on the significant impacts of host location and habitat in disease systems, emphasizing their importance and underscoring the critical need to integrate community ecology studies into disease research. By understanding how host location, habitat, and the broader environment shape microbial communities, we can gain valuable insights into disease dynamics, ultimately advancing our ability to manage and mitigate disease impacts effectively.

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INTRODUCTION

Multispecies assemblages of microbes within a particular habitat, or microbiomes (Whipps et al., 1988), are generally thought to behave like assemblages of macro-organisms (Berg et al., 2020; Buckley, 2003; Miller et al., 2018; Konopka, 2009). These assemblages can be disturbed when host conditions change, such the onset of disease (Dornelas, 2010). Just like with ecological assemblages of macro-organisms, such disturbances can affect the biodiversity and dynamics of the microbial community (Dornelas, 2010; Weinbauer & Rassoulzadegan, 2004). For example, viruses have the potential to alter the structure of a host's microbial community (Anderson et al., 2013; Koskella and Meaden, 2013; Gilbert et al., 2020; Mojica and Brussaard, 2014). Therefore, we must employ community ecology approaches to assess the impact of disease on host-associated microbial communities (Johnson et al., 2015).

Community ecology aims to understand the causes and consequences of species within a community (Johnson et al., 2015; Holyoak et al., 2005; Morin, 2009), which in the context of host-associated microbes would include interactions between the host, the overall microbial community and potential pathogens. The introduction of a pathogen or parasite operates as a disturbance, causing a shift in the microbial community and altering community interactions in two alternative trajectories in which the microbiota can (1) turn into an etiological agent or (2) mitigate the pathogen's impact (Weinbauer & Rassoulzadegan, 2004). Mechanistically, pathogens can trigger a cascade of events, directly or indirectly leading to a reduction in the abundance of dominant, competitive

bacteria, thereby creating an opportunity for other bacterial species to colonize the host. The process of pathogenic disturbance has been suggested to be equivalent to opening niche space, elevating overall bacterial diversity, and reshaping the entire community structure (Weinbauer & Rassoulzadegan, 2004). On the contrary, some pathogens reduce the diversity of microbial communities (Jiao et al., 2022; Jiang et al., 2019) potentially disrupting microbial community habitat filtering (Rogers et al., 2020). Whether disease leads to increased or decreased diversity of communities that protect or harm the host, we must account for these complex dynamics of host-microbiome-pathogen interactions within the context of its surrounding environment if we want to better understand the incidence and consequences of a disease.

The influence of the host's habitat and environment is rarely included in host-microbe-pathogen interactions and could have important impacts in communities with shifting niche space and habitat filters (Johnson et al. 2015; Prosser et al., 2007). For example, a community with lowered diversity creates an ecological niche primed for colonization of microbes from the surrounding environment (Bailey et al., 2010; Zuniga-Chaves et al., 2023). In contrast, a community with a stronger habitat filter must be colonized by “healthy” bacteria that can maintain viable bacterial populations during pathogen invasion (Munoz-Ucros et al., 2021). To address this knowledge gap at the interface of disease and community ecology, my study delves into the oyster microbial community, with a particular focus on how they are impacted by co-infections involving Ostreid herpesvirus (OsHV-1) and opportunistic bacterial pathogens, specifically *Vibrio*. To explicitly consider the influence of environmental conditions and potential colonists

on a microbial community, I examine the Pacific oyster (*Magallana gigas*) in the field and address the community dynamics both within the oyster and in its surrounding habitat.

Magallana gigas contends with OsHV-1 every summer during what are called 'summer mortality events' or 'Pacific Oyster Mortality Syndrome (POMS)'; these events can cause mortality levels up to 90% in a given population (de Lorgeril et al., 2018; Petton et al., 2015; King et al., 2019). Interestingly, in the absence of bacteria, a high viral load of OsHV-1 does not allow for full expression of the disease (King et al., 2019). Therefore, it is proposed that these summer mortality events are the result of a co-infection involving OsHV-1 and various *Vibrio* species (de Lorgeril et al., 2018). However, it is crucial to note that previous studies on POMS have primarily been conducted in laboratory settings, which omit a vital factor: the influence of the habitat location or natural environment on these intricate microbiome-pathogen interactions.

My thesis explores the essential role of community ecology in disease research, emphasizing the need for a holistic approach that considers the complex web of interactions among pathogens, hosts, and their environment. To account for environmental and site-based influences, I use a field-based approach to characterize microbial community dynamics in the presence of the disease. I compared healthy and infected oysters at two sites: one site known to have OsHV-1 present (Tomales Bay, CA) and one site where OsHV-1 has not been reported previously (Humboldt Bay, CA). I address four hypotheses based upon previous OsHV-1 research:

1. Microbial community dynamics will be affected by OsHV-1; specifically, the OsHV-1 infection will decrease microbial richness, diversity, and evenness.
2. OsHV-1 infected oysters will be more strongly influenced by bacterial colonization from the water column than healthy oysters because the presence of OsHV-1 will diminish the microbial community's ability to selectively control bacterial colonization from the environment.
3. POMS is thought to be a co-infection between OsHV-1 and *Vibrio;* therefore, I expect *Vibrio* abundance to increase with OsHV-1 load.
4. Because OsHV-1 will cause a shift in predominant bacteria, the microbial community composition of oysters infected with OsHV-1 will be dominated by *Vibrio*, while the relative abundances of other taxa will decline.

These hypotheses serve as a lens to investigate how bacterial communities respond to the presence of host disease, offering insights into diversity and compositional shifts in the context of habitat location influences. By testing these hypotheses, I aim to further integrate community ecology with disease ecology.

Study System

The number one most farmed invertebrate in the world, *Magallana gigas*, is prone to infection from OsHV-1, which can cause up to 90% mortality in farmed populations every summer during what are called summer mortality events or Pacific Oyster Mortality Syndrome (POMS) (Lorgeril et al., 2018; Petton et al., 2015; King et al., 2019).

In wild populations, *M. gigas* provide many benefits to marine and estuarine populations including food, protection from predators, buffers from environmental stress, and denitrification (Vezzulli et al., 2015; DePiper et al., 2017). In addition to these key roles in the ecosystem, oysters are also valuable aquaculture products. Worldwide, oysters make the largest contribution to the farmed seafood industry by contributing 4.7 million tons harvested per year (NOAA).

Studies have shown that disease progression begins with the infection of OsHV-1 causing a change in the oyster's microbiota (de Lorgeril et al., 2018). These changes have etiological effects, primarily with bacteria from the genus *Vibrio*. *Vibrio sp.* then become secondary opportunistic pathogens within the oyster, amplifying the effects of viral infection. In the absence of bacteria, a high viral load of OsHV-1 may not kill the oyster, supporting the hypothesis that summer mortality events are caused by a co-infection of OsHV-1 and varying *Vibrio* species (King et al., 2019).

Although laboratory experiments allow us to understand the fundamentals of this co-infection, however, none of them include source location analyses, meaning they do not assess the location of the oysters nor the microbial community of the surrounding environment, nor do they use samples collected directly from the field. Lack of site analyses, i.e. accounting for location of the oyster in the field and microbial community of the surrounding environment, in these studies leaves out key information on site influences on this host-pathogen interaction. The influence of the host's habitat and environment could have important impacts in communities with shifting niche space and

habitat filters and must therefore be included to fully understand the development of the disease (King et al., 2019; Johnson et al., 2015).

METHODS

To evaluate and compare the microbial communities of oysters with and without OsHV-1, I collected *M. gigas* and water samples from two locations: one known to have OsHV-1 present (Tomales Bay, CA) and one that does not (Humboldt Bay, CA) (Fig. 1).

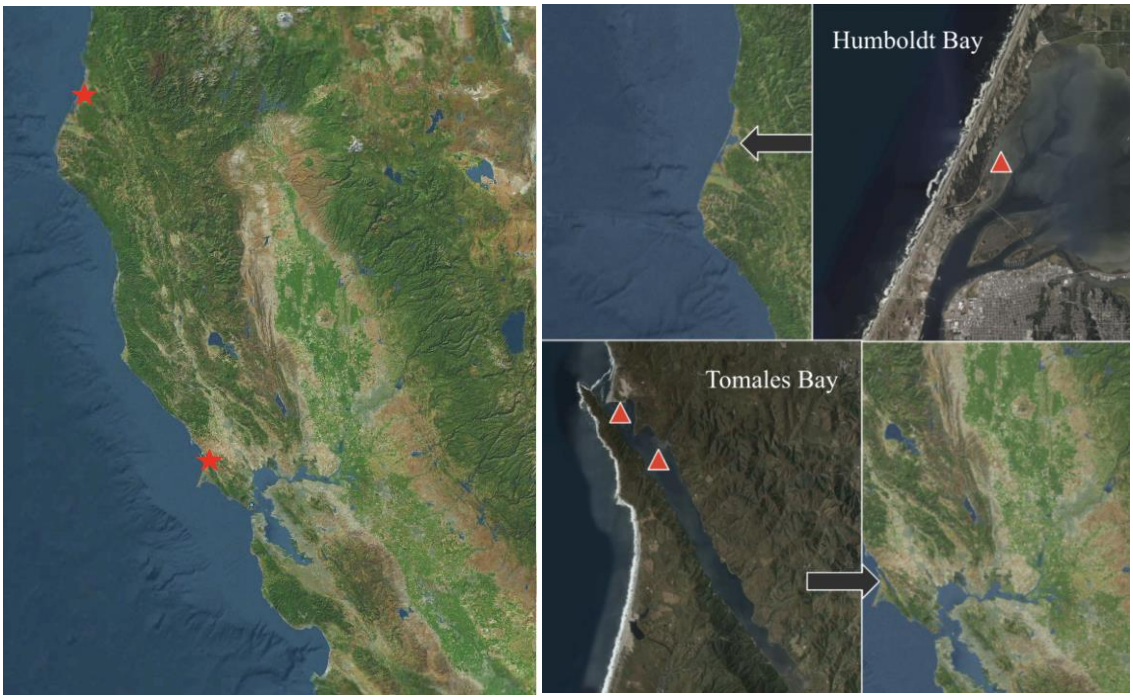


Figure 1. Map of the California coast (left). Collection sites (right): Humboldt Bay (top right) and Tomales Bay (bottom left). Specific collection locations indicated by red triangles.

Preparation for all Hypotheses

Oyster collection and dissection

I collected 39 feral *M. gigas* from the Humboldt Bay marsh in Manila, CA (40.83262° N, 124.17119° W) and 25 *M. gigas* from Humboldt Bay Oyster Company in

July 2022 (54 total Humboldt Bay oyster samples). Once collected from the site, I brought the oysters back to the Cal Poly Humboldt preparation laboratory. I collected potentially infected *M. gigas* from Hogg Island Oyster company and two locations in Tomales Bay, CA (38.20959° N, 122.93172° W; 38.22892° N, 122.95906° W) during two mortality events in August 2022 (103 total Tomales Bay oyster samples). To increase the chances of collecting an infected oyster, I selected oysters in bags with moribund oysters, meaning that several oysters were open. I processed and dissected all oysters collected in Tomales Bay at the Shellfish Pathology Lab at Bodega Bay Marine Lab in Bodega Bay, CA. I rinsed all oysters with tap water to remove sediment and various algae and invertebrates attached to the valves. Following rinsing, I sprayed all oysters thoroughly with 70% ethanol to kill any surface bacteria and weighed them. I dissected the oysters under a sterilized laminar flow hood, using sterile dissection tools. I removed the adductor muscle and gill tissues, sterilizing tools between tissue types to minimize contamination. The distinct appearances of each tissue type allow easy detection of any contamination. I placed dissected tissues in sterile 1.5 mL microcentrifuge tubes with ethanol to preserve the microbial community during transport. I then stored all tissue samples at -80 °C.

I also collected 50 mL of water at each collection site at both the Humboldt Bay marsh and Tomales Bay and stored samples in -80 °C. Collection site for water was largely dependent on tide. In Humboldt Bay, water was collected approximately 1-2 meters from the oyster collection site (3 total Humboldt Bay water samples collected). In

Tomales Bay, water was collected approximately 3 meters from the oyster collection site (5 total Tomales Bay water samples).

Oyster water and sample DNA extraction

Prior to any lab work done on my samples, I performed preliminary DNA extractions and 16S sequencing to test the effectiveness of ZYMOBIOMICS miniprep kits and how each tissue type responded to 16S sequencing. Following oyster collection and dissection and water collection, I performed microbial DNA extractions on all samples, except the adductor muscle tissue. I used the DNA extracted from the adductor muscle tissue as a template to amplify the COI gene region to determine the species of oyster samples (Cordes et al., 2008). However, I used gills for analyzing the oyster microbial community, because it is the conventional method for microbial community analyses as gills exhibit the highest degree of interaction with their surrounding environment (Logeril et al., 2018; Pathirana et al., 2019; King et al., 2019; Petton et al., 2015). Prior to DNA extraction, I cut and weighed between 0.015g and 0.050g for both adductor muscle tissue and gill tissue. Following preprocessing, I performed DNA extractions on all samples using the ZYMOBIOMICS miniprep kit following the manufacturer's protocol. I then quantified DNA yield using the Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer.

To prepare marine water samples for DNA extraction, I passed samples through a 0.22 µm mixed cellulose ester (MCE) membrane filter using a 60 mL syringe. I then cut the filters in half and placed them in ZR BashingBead Lysis tubes. I performed DNA

extractions using the ZYMOBIOMICS miniprep kit with the following changes to the manufacturer's protocol: I inverted spin columns after each wash buffer step, a “dry spin” was done after second wash buffer 2 stage, and I eluded DNA in 20 µL of Low EDTA buffer prewarmed to 60°C in place of DNase RNase free water (Murguia et al., 2021). Following DNA extraction, I quantified DNA yield using the Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer.

Species determination of feral oyster samples

I determined the species of feral oysters collected at the Humboldt Bay marsh in Manila CA by performing PCR and gel electrophoresis. I used total genomic DNA from adductor muscle tissue for amplification. I performed PCR amplification of the COI gene region using primers LCOI 490, Fw- GGTCAACAAATCATAAAGATATTAGG and HC02198, Rev- TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al. 1994). LCOI 490 and HC02198 were both present at a concentration of 100 µM. Fifty µl PCR reactions occurred under the following conditions: denaturation for 3 minutes at 95°C followed by 30 cycles of 1 minute at 95°C, 2 minutes at 52°C, two minutes at 72°C, and a final extension for 5 minutes at 72°C (Cordes et al., 2008). Following amplification, I performed standard agarose gel electrophoresis and compared band sizes, ~700 bp, to a farmed *M.gigas* sample that was amplified alongside unidentified sample (Cordes et al., 2008).

Oyster and water sample amplicon sequencing and sequence processing

I sent DNA samples to Argonne National Laboratory for 16S rRNA amplicon sequencing using the MiSeq Illumina (San Diego) platform. I targeted the V4 region of the 16S rRNA gene (primers 341F 805R) and followed the protocol described by the Earth Microbiome Project (Caporaso et al., 2010). Briefly, all DNA samples were standardized to the lowest concentration by diluting with ultrapure water, plated in 96 well plates, and shipped.

I demultiplexed bacterial sequences using the program *idemp* (Blostein et al., 2020). Following demultiplexing, I performed all analyses in the R coding environment (version 4.1.1, R Core Team, 2020). I processed raw amplicon sequencing data through *dada2* (version 1.22.0, Callahan et. al., 2016) following the default parameters. Taxonomic affiliation was generated using the Greengenes database (McDonald et. al., 2011) using a 97% similarity threshold. Following *dada2* processing, I used *phyloseq* (version 1.38.0, McMurdie & Holmes, 2013) *dplyr* (version 2.3.3, Wickham et al., 2019), *vegan* (version 2.6-4, Oksanen, 2007), *microbiome* (version 1.16.0, Lahti, 2018), and *ggplot2* (version 3.4.2, Wickham, 2011) packages for statistical analyses.

Quantitation of bacterial and viral load in gill tissue

I quantified the levels OsHV1 and *Vibrio* in my samples with quantitative PCR (qPCR) using the QuantStudio™ 3 Real-Time PCR System using the following cycling program: enzyme activation at 95 °C for 10 seconds and 40 cycles of denaturation at 95°C for 20 seconds, hybridization at 62.3°C for 20 seconds and elongation at 72°C for 25 seconds. The total volume of all reactions was 20 µL. One qPCR targeted the catalytic

subunit ORF 100 (a catalytic subunit of polymerase δ) of the OsHV1 genome by using the primers ORF 100 F - TGATGGATTGTTGGACGAGA and ORF 100 R - ATCACATCCCTGGACGCTAC (Divilov et al., 2019) at a concentration of 100 µM. This qPCR consisted of 10 µl Luna Universal Master Mix, 0.8 µl forward primer, 0.8 µl reverse primer, 5.9 µl H_2O, 0.5 µl bovine serum albumin (400 ng/ µl), and 2 µl template DNA per reaction. OsHV-1 DNA was used as a positive control and standard curve was made using a plasmid containing OsHV-1 DNA from 3 to 3 x 10^7 copies per reaction. Both the positive control and standard curve were procured from Dr. Colleen Burge at the Shellfish Pathology Lab in the Bodega Bay Marine Lab at Bodega Bay, CA.

Quantitative PCR targeting *Vibrio* consisted of 0.05 µl forward primer, 0.05 µl reverse primer, 10 µl Luna Universal Mastermix, and 8.9 µl H_2O per reaction. The total volume per reaction was 20 µl. Total *Vibrio* group specific primer pairs targeting a variable region of the 16S rRNA gene were 567F 5'-GGCGTAAAGCGCATGCAGGT-3' and 680R 5'GAAATTCTACCCCCCTC TACAG-3' at a concentration of 100 µM (Westrich et al., 2018). *Vibrio fischerii* was used as a positive control and standard curve from 38 to 3.8 x 10^7 copies per reaction. The cycling program was as follows: enzyme activation at 95 °C for 10 seconds and 40 cycles of denaturation at 95°C for 20 seconds, hybridization at 62.3°C for 20 seconds and elongation at 72°C for 25 seconds. Reactions were amplified using the QuantStudio™ 3 Real-Time PCR System.

Hypotheses 1, 2, and 4 use 16S sequencing data. I obtained a total of 78,378 reads, for an average of 496 read counts per sample, which were clustered in

33479 ASVs. I discarded 23,048 ASVs because of low read counts (< 0.00005 reads). Based on qPCR results, 28 of 103 Tomales Bay oysters were infected with OsHV-1.

Data Analyses

Hypotheses 1

To establish the effect of site and type (describes the type of sample: healthy, viral, or water) on oyster microbial community dynamics, I calculated three metrics of diversity: Shannon's index, evenness, and richness. Shannon's index was calculated by using the "diversity 'Shannon'" function in R which follows the equation: $H' = \sum_i p_i \log_b p_i$, where p_i is the proportional abundance of species *I*, and *b* is the base of the algorithm (Fisher et al., 1943; Hurlbert, 1971; Jost, 2007). Due to the unbalanced nature of this study where infected samples were only present in one site, I separated samples into two categories for analysis: (a) all healthy samples and their water (to compare the microbial communities within oysters and to water samples across sites), and (b) all Tomales Bay samples (to compare between healthy and diseased oysters). In group (a), I used a GLM, Wald test, and Bonferroni correction to assess significant effects of site and type and their interaction on diversity metrics. In group (b), I performed an ANOVA to assess any significant effects of type on richness, Shannon Index, and evenness, followed by a Tukey Post-Hoc test to determine which groups were significantly different from one another.

To further investigate the potential effect of type and site on microbial community composition, I performed a perMANOVA on groups (a) and (b). Because there is only

one independent variable (type) in group (b), I followed the perMANOVA with a permutation test for homogeneity of multivariate dispersions. To illustrate these patterns in community composition, I used non-metric multidimensional scaling (nMDS) based on Bray-Curtis dissimilarities using the *vegan* package (version 2.6-4, Oksanen, 2007).

Hypothesis 2

To determine if viral presence affects the microbial community's ability to filter its environment, I performed a beta dispersion followed by a permutation test. In analyzing the predominant taxa at each site and within various types, I employed R (version 4.1.1, R Core Team, 2020) and used base R along with *dplyr* (version 2.3.3, Wickham et al., 2019) to filter ASVs, excluding those occurring less than 0.005 times. I then generated rank abundance plots. Based on this analysis, I compiled a table illustrating the most abundant genera specific to each site and type.

Hypothesis 3

To analyze *Vibrio* abundance and OsHV-1 load, I processed qPCR quantities using the R studio base code. I used a linear regression to establish the relationship between *Vibrio* abundance and viral load. Then I used the *F*-test from the lm.summary() function in R to test for significance of this relationship.

Hypothesis 4

In analyzing the predominant taxa at each site and within various types, I employed R (version 4.1.1, R Core Team, 2020) and used base R along with *dplyr* (version 2.3.3, Wickham et al., 2019) to filter ASVs, excluding those occurring less than

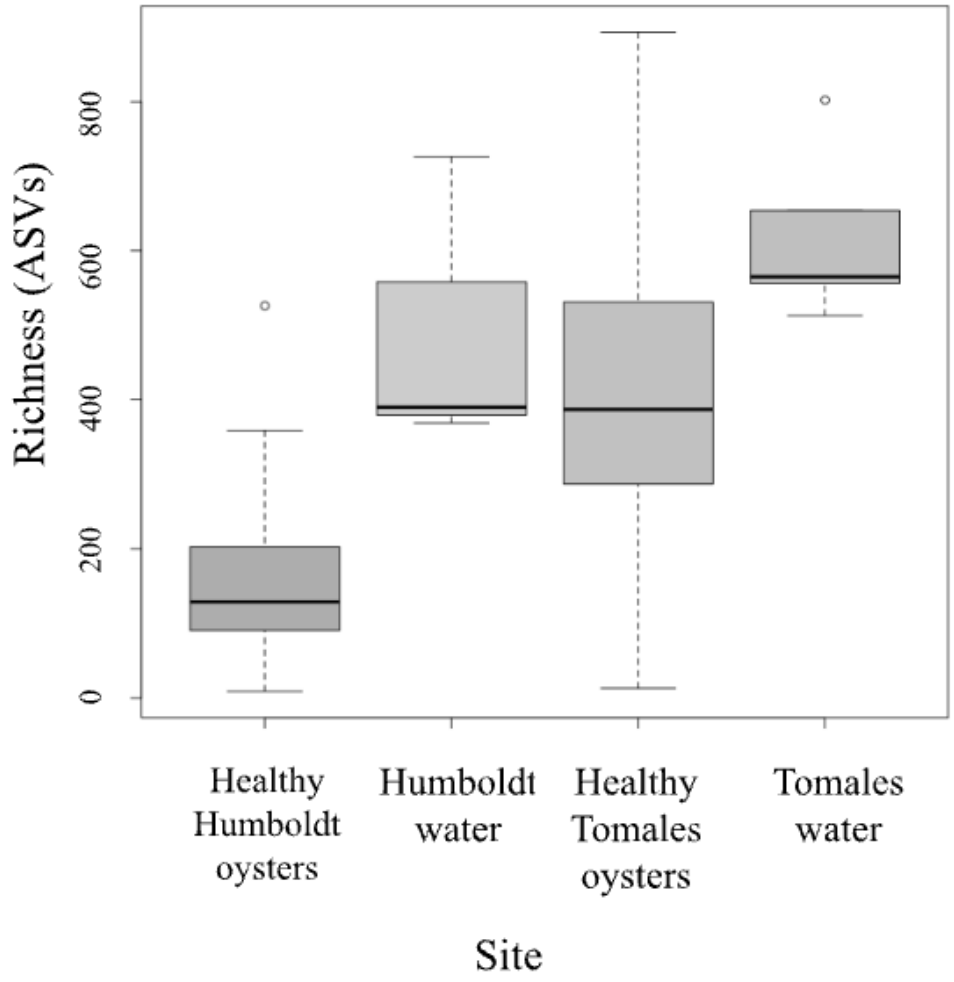
0.005 times. I then generated rank abundance plots. Based on this analysis, I compiled a table illustrating the most abundant genera specific to each site and type.

RESULTS

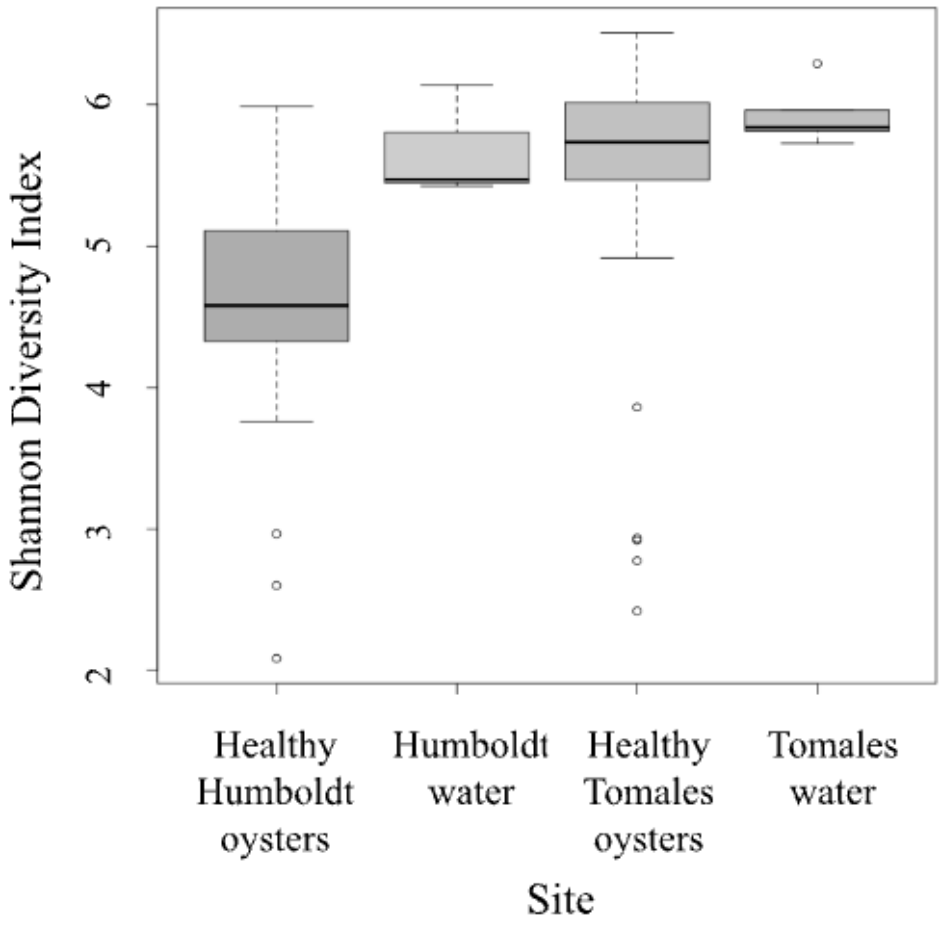
Comparison of Microbial Community Dynamics in Healthy and Infected Hosts

In group (a), healthy Humboldt oysters displayed lower microbial community richness and Shannon Index compared to Humboldt Bay water and Tomales Bay oyster and water samples (Fig. 2a, b). The richness of the microbial community varied significantly among samples from different sites and sample types (Table 1). This variation indicates the sample type's notable influence on microbial community richness. Additionally, the Shannon Index of the microbial community was significantly impacted by site but not by the type of sample (Table 1). Evenness was influenced by only sample type, with oyster samples exhibiting a higher level of evenness compared to water samples (Table 1 and Fig. 2c). I observed an interaction between type and site regarding microbial community evenness, however, it is possible this was due to the unbalanced design; primarily, more oyster samples than water (Table 1). Moreover, both site and type had a statistically significant effect on the community composition of the oyster microbial community and an interaction between type and site was observed (Table 2).

a.


Richness (ASVs)
0
200
400
600
800
Healthy Humboldt oysters
Humboldt water
Healthy Tomales oysters
Tomales water
Site

b.


Shannon Diversity Index
2
3
4
5
6
Healthy Humboldt oysters
Humboldt water
Healthy Tomales oysters
Tomales water
Site

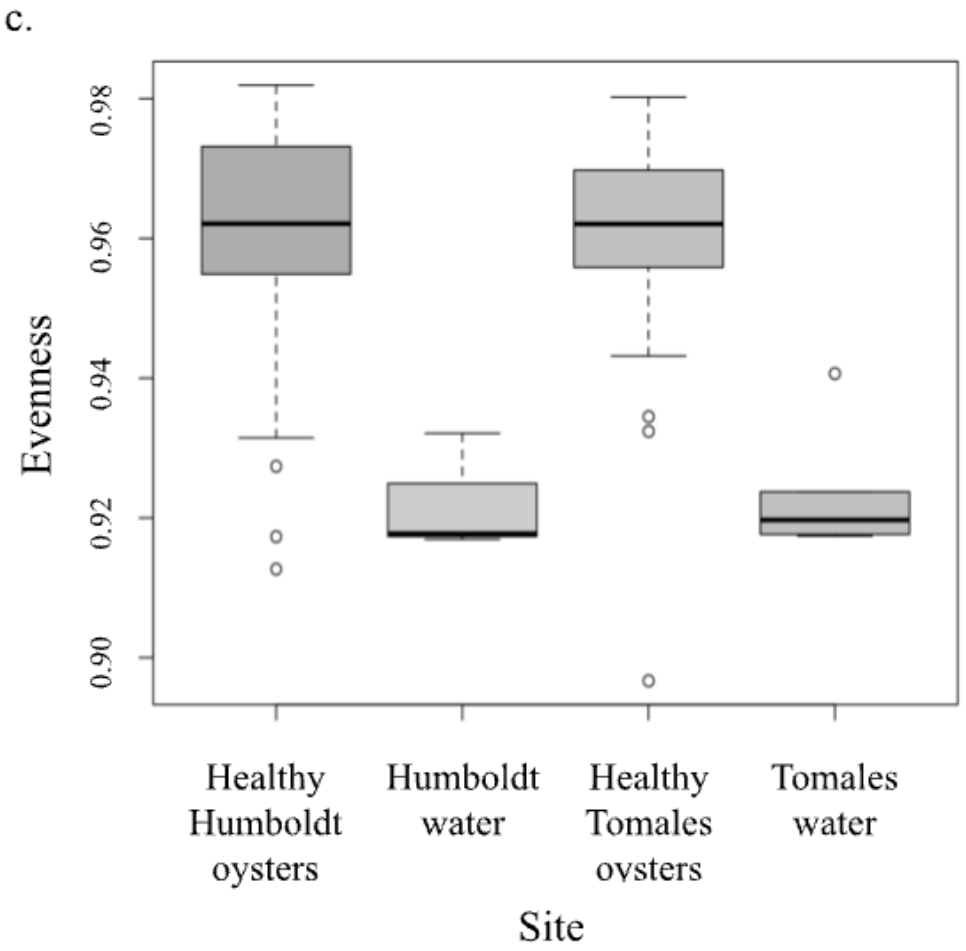


Figure 2. (a) Microbial community richness of Tomales Bay healthy oysters and water and Humboldt Bay healthy oysters and water samples. Box plots display median (horizontal line) and outlier values. (b) Shannon Diversity Index of healthy oysters and water samples at both sites: Humboldt Bay and Tomales Bay. Box plots display median (horizontal line) and outlier values. (c) Microbial community evenness of healthy oysters and water samples across sites. Box plots display median (horizontal line), lower and upper quartiles (fences), where 50% of data is found (box) and outliers (open circles).

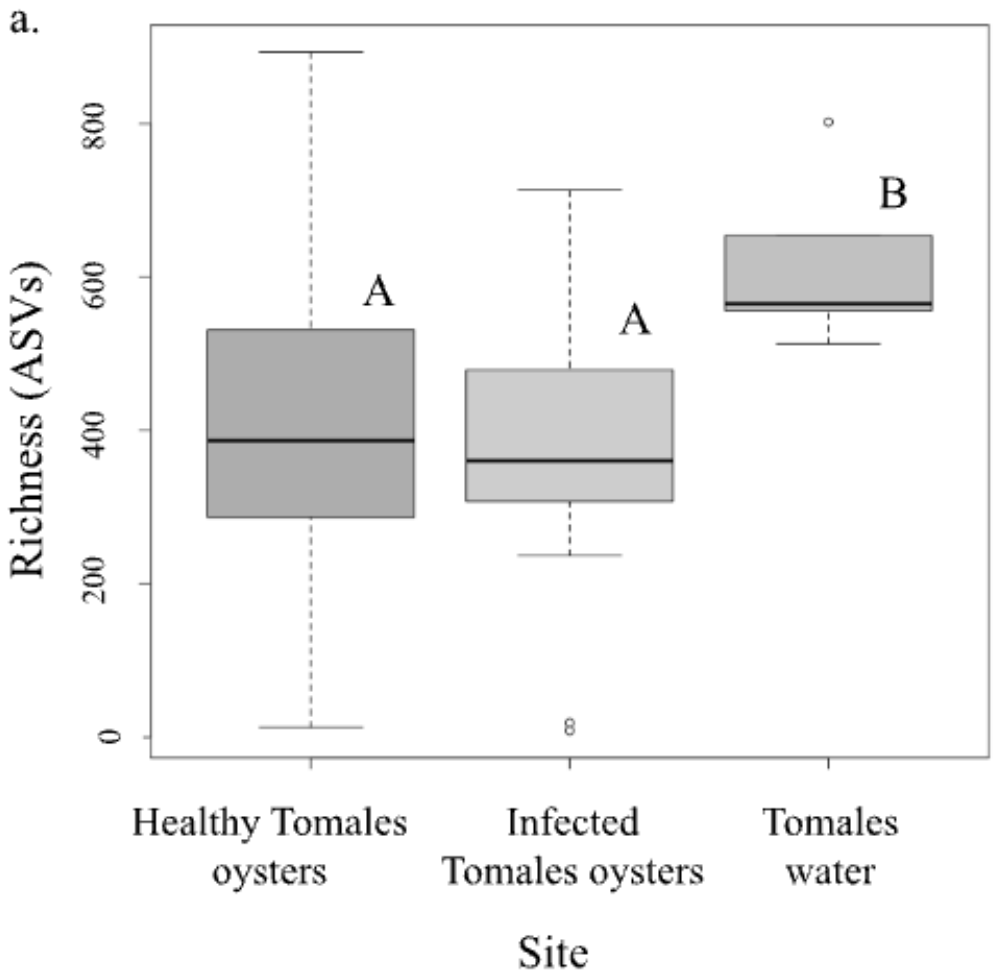
Table 1. Results of a GLM and Wald's test with Bonferroni correction showing the effect of site (location of collection) and sample type (healthy or water) on richness, evenness, and Shannon-Wiener Index of microbial communities. Bold *P*-values indicate statistically significant effects.

	Predictors	LR Chiseq	Df	*P*-value	*P*-value adj
a. Richness	Type	11.213	1	**8.12×10^{-4}**	**8.12×10^{-4}**
	Site	85.3	3	**9.16×10^{-15}**	**1.83×10^{-14}**
	Type:Site	85.3	3	**2.2×10^{-18}**	**6.64×10^{-18}**
b. Shannon-Wiener Index	Type	51.4	3	0.0587	5.87×10^{-2}
	Site	51.4	3	**3.76×10^{-11}**	**1.13×10^{-10}**
	Type:Site	51.4	3	**3.98×10^{-11}**	**1.13×10^{-10}**
c. Evenness	Type	56.1	1	**7.05×10^{-14}**	**2.11×10^{-13}**
	Site	55.2	3	0.94	9.5×10^{-1}
	Type:Site	55.2	3	**6.16×10^{-12}**	**1.23×10^{-11}**

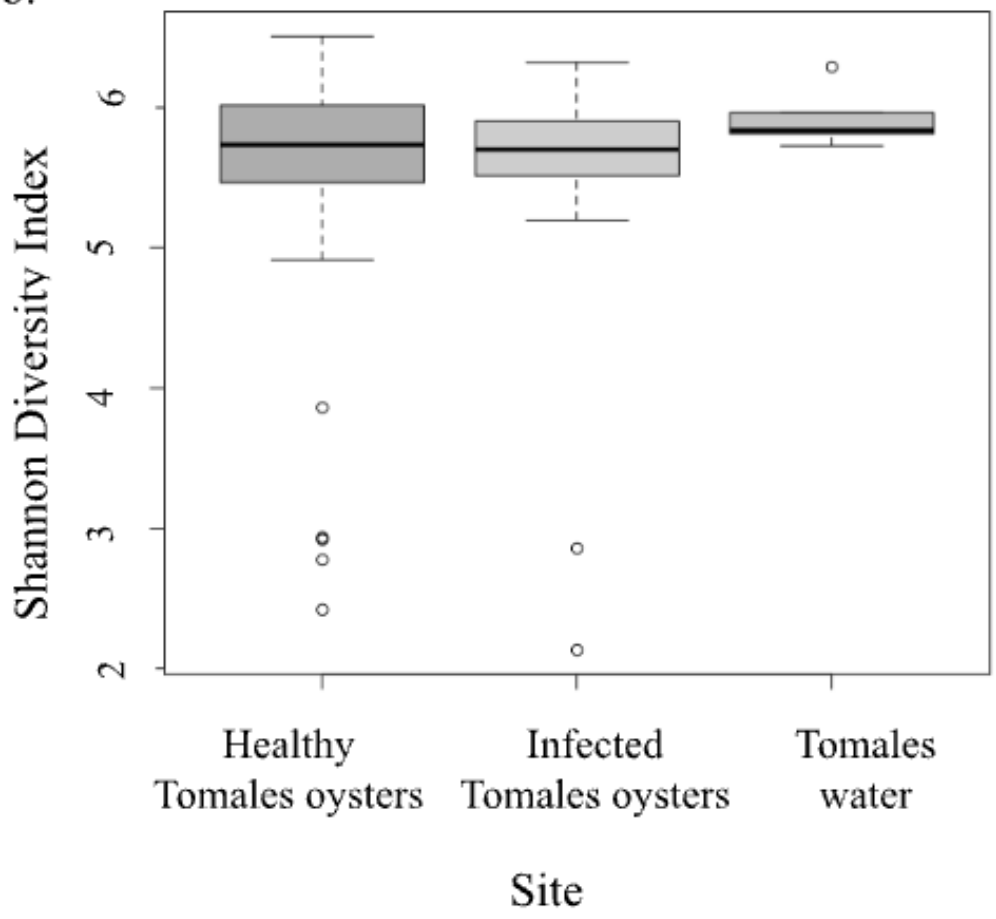
Table 2. Results of a perMANOVA showing the effect of site (location of collection) and sample type (healthy or water) on microbial community composition of Tomales Bay oysters and water and Humboldt Bay oysters and water. Bold *P*-values indicate statistically significant effects.

Predictors	Df	Sum Sq	R2	F value	*P-value*
Type	1	1.76	0.03	**4.85**	**0.001**
Site	2	3.65	0.07	**5.03**	**0.001**
Type:Site	3	5.42	0.11	**4.97**	**0.001**
Residuals	126	45.78	0.89		
Total	129	51.19	1.00		

In group (b), both healthy and infected Tomales Bay oysters, as well as water microbial communities showed similar richness and Shannon Index (Fig. 3a, b). Richness was notably influenced by microbial community type, with water showing a higher value (Table 3; Fig. 3a), mirroring the trend observed in group (a). Shannon index, remained unaffected by the microbial community type (Table 3). Notably, evenness was significantly lower in water samples compared to the oyster microbial community (Fig. 3c), with the former being influenced by the sample type (Table 3). Additionally, relative abundance of microbial communities in both healthy and infected Tomales Bay oyster samples, as well as water samples were significantly impacted by the sample type, particularly water (Table 5; Fig. 4).


a.
Richness (ASVs)
0
200
400
600
800
A
A
B
8
Healthy Tomales oysters
Infected Tomales oysters
Tomales water
Site

b.


Shannon Diversity Index
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Healthy Tomales oysters
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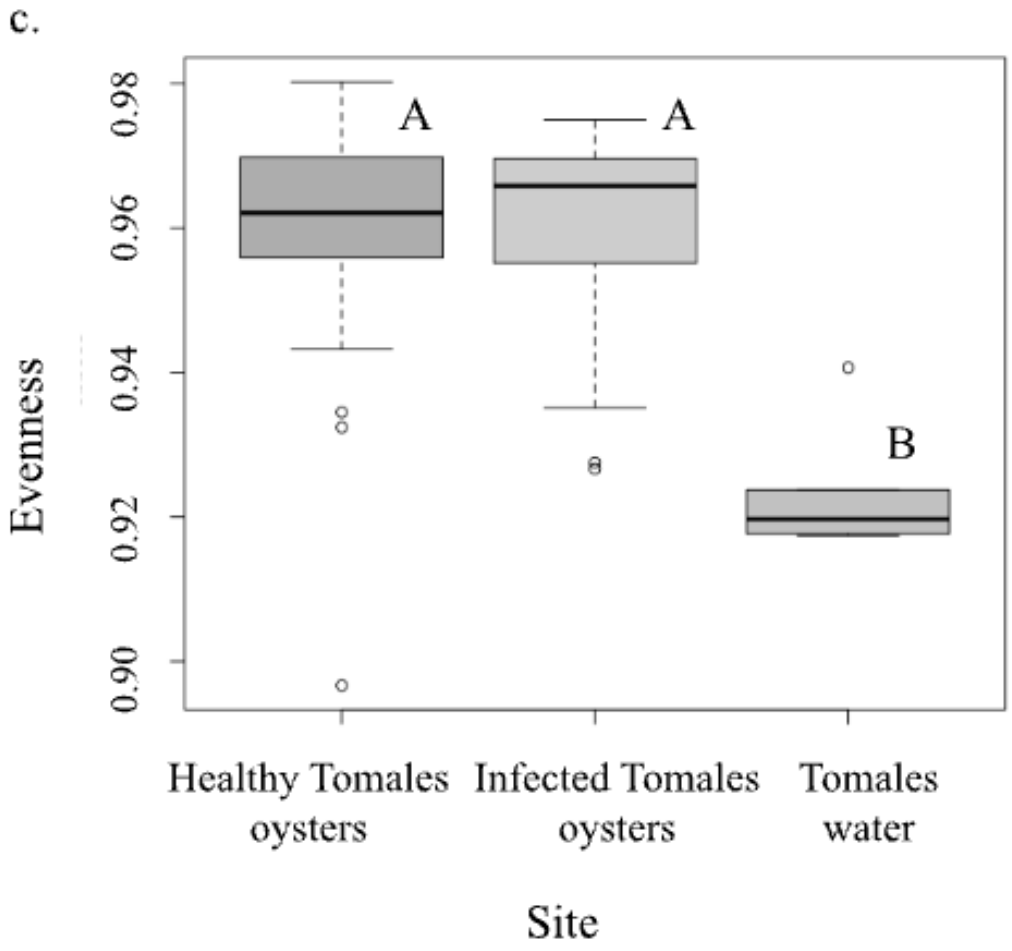

Figure 3. (a) Microbial community richness's in each type (healthy, infected, and water) of Tomales Bay samples. Letters indicate results from Tukey Post Hoc test. Box plots display median (horizontal line) and outlier values. (b) Microbial community Shannon Diversity Index in each type (healthy, infected, and water) of Tomales Bay samples. There are no significant differences between groups. Box plots display median (horizontal line) and outlier values. (c) Microbial community Shannon Diversity Index in each type (healthy, infected, and water) of Tomales Bay samples. Box plots display median (horizontal line) and outlier values.

Table 3. Effect of type (healthy, infected, and water) on microbial community richness, Shannon index, and evenness on microbial community of Tomales bay oyster and water samples. Bold *P*-values indicate statistically significant effects. Bold *P*-values indicate statistically significant effects.

	Predictors	LR Chiseq	df	*P*-value
a. Richness	Type	6.69	2	**0.035**
b. Shannon Index	Type	1.19	2	0.5514
c. Evenness	Type	38.4	2	**<0.001**

Table 4. PerMANOVA results indicating effect of type on microbial community composition of Tomales Bay oyster and water samples. Bold *P*-values indicate statistically significant effects. The R^2 indicates multivariate variance explained.

Predictors	df	Sum Sq	R^2	*F*-value	*P*-value
Type	2	1.59	0.042	**2.29**	**0.001**
Residuals	105	36.6	0.96		
Total	107	38.2	1.00		

Table 5. Results of permutation test. Pairwise comparisons (observed p-value below diagonal, permuted *P*-value above diagonal). Bold *P*-values indicate statistically significant effects.

	Healthy	Viral	Water
Healthy		**5.00×10^{2}**	**0.001**
Viral	7.21×10^{2}		**0.001**
Water	6.99×10^{14}	6.65×10^{7}	

Type had significant effects on community composition in Tomales Bay samples (Table 4). This significance is derived from both water and viral presence (Table 5). Regardless of water samples skewing results, the pattern of site influencing microbial composition consistently in oyster samples remains clear (Fig. 4), while Tomales Bay oyster diversity patterns remained indistinguishable throughout all statistical analyses. (Fig. 3a, b, c, and Fig. 4).

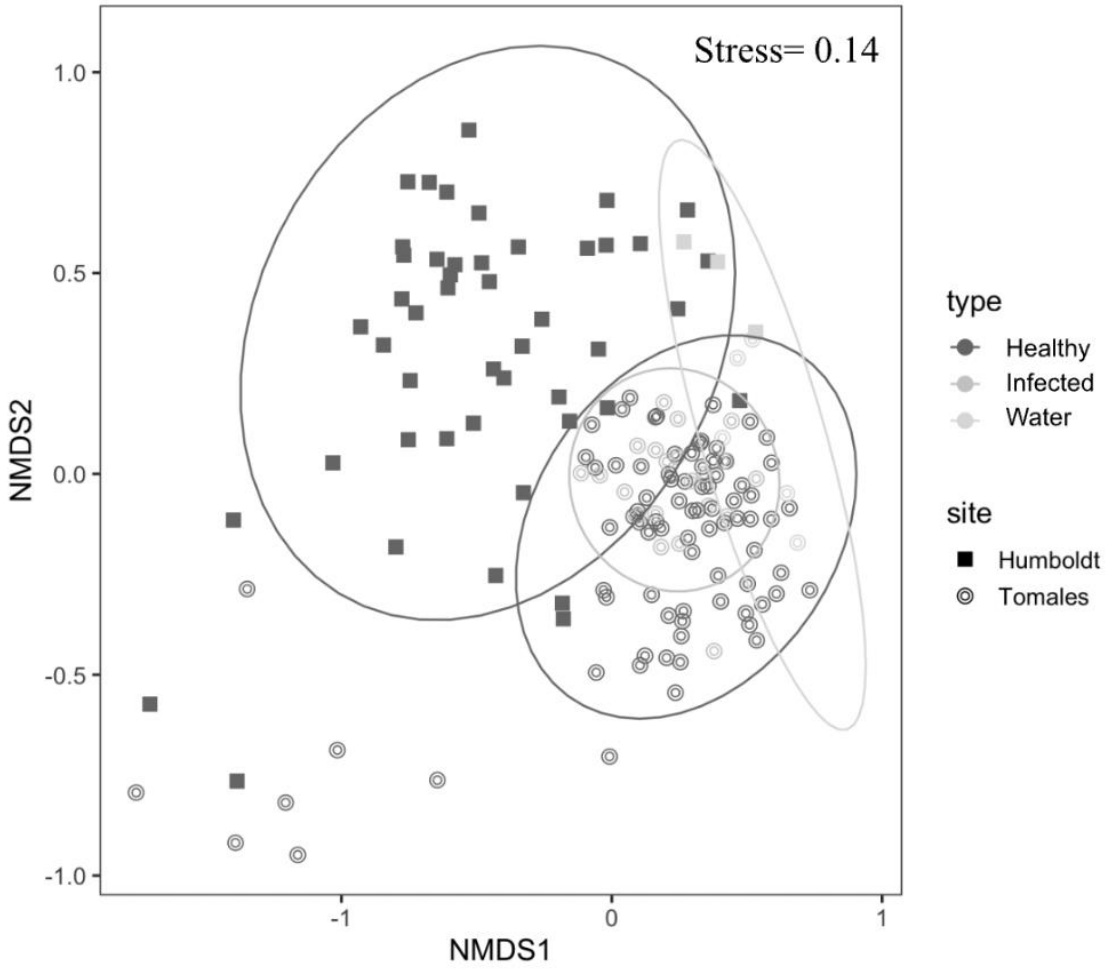

Figure 4. Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis dissimilarities derived from ASVs at the level for site and type. Site, Humboldt Bay and Tomales Bay, are indicated by squares and open circles, respectively. Type, healthy, infected, and water, are color coded.

Effects of OsHV-1 Infection on Environmental Filtering

Infected oysters were not strongly influenced by bacterial colonization from the water column compared to healthy oysters and presence of OsHV-1 did not diminish the microbial community's ability to filter the environment (Fig. 5, Table 6). Significant differences between microbial communities from the oyster and those from the water column indicate a resistance to colonization from the water column. If the oyster

communities became more like water bacteria when infected, that would indicate susceptibility to colonization from the water column.

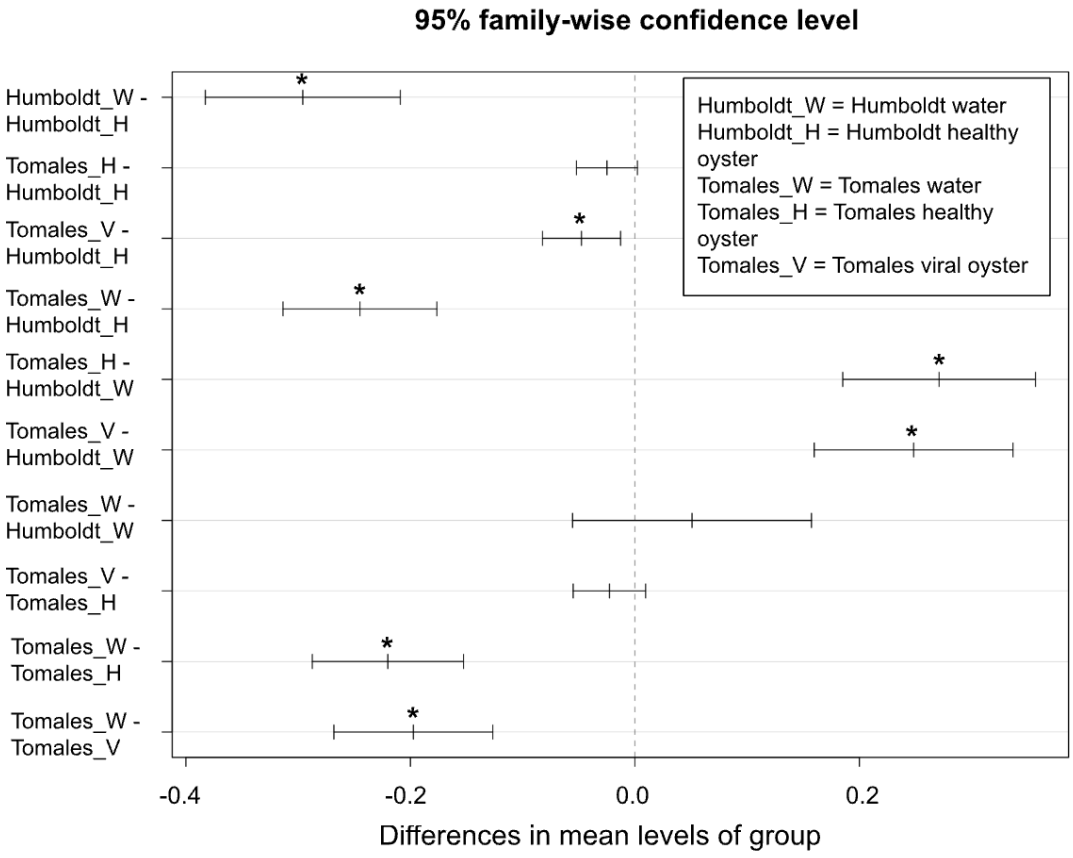

Figure 5. Results of pairwise comparisons of relative abundances at all sites (Humboldt Bay and Tomales Bay) and with all sample types (healthy, infected, and water). Pairs with similar microbial communities, representing sufficient environmental filtering, indicated with an asterisk.

Table 6. Results of permutation test. Pairwise comparisons (observed *P*-value below diagonal, permuted *P*-value above diagonal):

	Humboldt_H	Humboldt_W	Tomales_H	Tomales_V	Tomales_W
Humboldt_H		1.000×10^{-2}	1.000×10^{-2}	1.000×10^{-2}	0.01
Humboldt_W	3.703×10^{-15}		1.000×10^{-2}	1.000×10^{-2}	0.29
Tomales_H	7.395×10^{-3}	2.840×10^{-13}		7.000×10^{-2}	0.01
Tomales_V	3.639×10^{-4}	8.713×10^{-7}	7.215×10^{-2}		0.01
Tomales_W	7.310×10^{-16}	2.495×10^{-1}	6.989×10^{-14}	6.658×10^{-7}	

Vibrio Abundance and Infection Relationship

Viral load and *Vibrio* abundance were not significantly correlated (Fig. 6). To compare these results with other studies, it is important to note that the highest genomic units (gu) of viral DNA was 6, the minimum was 0.43 gu and the average was 2.51 gu. Similarly, the highest gu of *Vibrio* in this study was 4.2×10^6 in infected oysters.

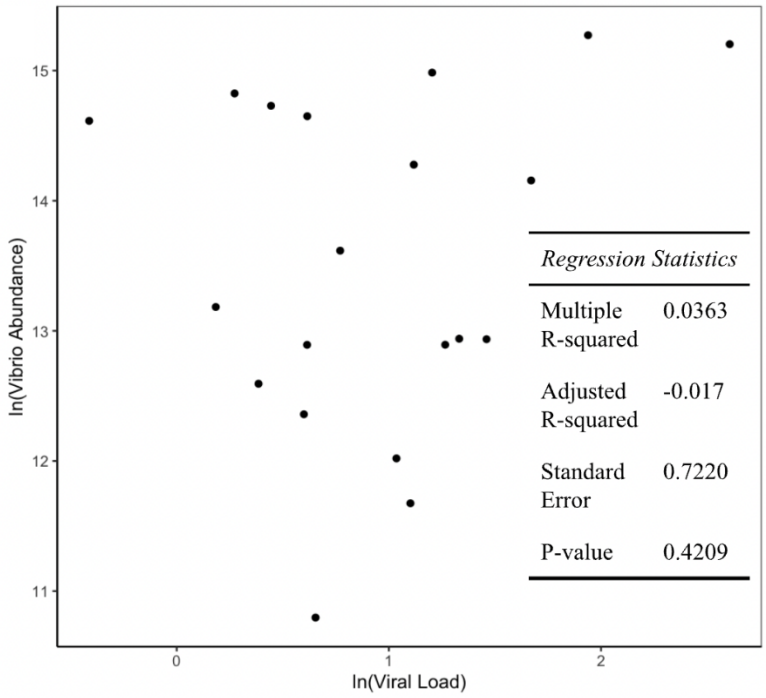

Figure 6. Results of a linear regression showing an insignificant relationship between OsHV-1 and *Vibrio* abundance (*P*= 0.421). Both abundance of OsHV-1 and *Vibrio* are expressed as the natural log of genomic units per 1 µl of total DNA extraction.

Oyster Microbial Community Composition

Contrary to my expectations, oyster microbial community composition was not dominated by *Vibrio* (Table 7a, 7b, Appendix 1). *Vibrio* was in the top 10 microbes present in only healthy oysters at both sites- Humboldt and Tomales, but not present in infected oyster samples. The genera that occurred in all four sample types were *Arcobacter*, *Halioglobus*, and NS5_marine_group. Unique genera to infected Tomales bay samples include *Pleurocapsa PCC 7319* and *Polaribacter*.

Table 7a. Results of the 10 most abundant genera for oysters at each site (Humboldt Bay and Tomales Bay) and type (healthy and infected). Similar genera among samples indicated in bold.

	Humboldt healthy oysters	Tomales healthy oysters	Tomales infected oysters
1	***Arcobacter***	*Altererythrobacter*	***Arcobacter***
2	***Halioglobus***	*Aquimarina*	*Clade_Ia*
3	*Marinobacterium*	***Arcobacter***	*Colwellia*
4	*Mycoplasma*	*Clade_Ia*	***Halioglobus***
5	**NS5_marine_group**	*Colwellia*	*Loktanella*
6	*Polynucleobacter*	***Halioglobus***	**NS5_marine_group**
7	*Pseudomonas*	*Loktanella*	Pleurocapsa_PCC-7319
8	*Stenotrophomonas*	**NS5_marine_group**	*Polaribacter_4*
9	*Sulfurovum*	*Polynucleobacter*	*Polynucleobacter*
10	*Vibrio*	*Vibrio*	*Sulfurovum*

Table 8b. Results of the 10 most abundant genera for water at each site (Humboldt Bay and Tomales Bay). Similar genera among samples indicated in bold.

	Tomales water samples	Humboldt water samples
1	***Arcobacter***	***Arcobacter***
2	Candidatus *Actincomarina*	Candidatus *Aquiluna*
3	Candidatus *Punicespirillum*	Candidatus *Punicespirillum*
4	Clade_Ia	Clade_Ia
5	***Halioglobus***	*Glaciecola*
6	ML602J-F1	*Marinobacterium*
7	**NS5_marine_group**	NS3a_marine_group
8	OM43_clade	**NS5_marine_group**
9	*Polaribacter_4*	OM43_clade
10	*Vibrio*	*Planktomarina*

DISCUSSION

My thesis provides strong evidence that the location and habitat of the host has significant effects on disease systems and, therefore, should be included in when studying disease dynamics in natural systems. I found that microbial community dynamics were impacted by OsHV-1 infection and location, yet the presence of OsHV-1 did not result in decreased richness, diversity, or evenness in the microbial community. Furthermore, OsHV-1 infection did not inhibit the oyster microbial community's ability to filter its environment, and, contrary to expectations, *Vibrio* abundance did not exhibit a significant increase with OsHV-1 load. Similarly, the microbial community in infected samples did not exhibit dominance by *Vibrio* and instead, *Arcobacter*, a common marine microbe, was found to be the most abundant.

Microbial Community Dynamics in Relation to OsHV-1

When comparing all healthy samples from Tomales Bay and Humboldt Bay alongside water samples (group (a)), significant variations was observed in all diversity metrics (richness, Shannon Diversity Index, and evenness), highlighting the influence of source location. Furthermore, diversity metrics were not impacted by infection status in Tomales Bay oyster samples (group (b)), contradicting prior research (de Lorgeril et al., 2018; Pathirana et al., 2019) that suggested that there is lower bacterial diversity in infected oysters. The apparent lack of effect of OsHV-1 infection on microbial diversity in my study may attributed to specific site conditions such as water temperature. The

narrower temperature range in Humboldt Bay (11-14°C) compared to Tomales Bay (10-20°C) means Humboldt Bay experiences more consistent temperatures. This stability may reduce stress on marine organisms and contribute to its lower susceptibility to summer mortality events compared to Tomales Bay, which undergoes wider temperature fluctuations. Along with this, my results are concordant with a previous study on *Crassostrea virginica* (Ossai et al., 2017) that demonstrated that microbial communities are significantly influenced by the source location of the habitat. It remains unclear what factors contribute to such a low diversity in these invertebrates.

While microbial communities from both sites exhibited comparable evenness, water samples displayed lower levels than oyster samples. A potential reason for higher evenness in oyster-associated bacteria is the influences of the parental microbiome colonizing early larval stage bacteria, creating a consistent and even microbial community regardless of site (Unzueta-Martínez et al., 2022). In contrast, past research on water microbiomes has linked lower evenness to factors such as salinity, temperature, organic matter, and pH (Hou et al., 2017).

Community composition was significantly affected by collection site of the sample. Differences in composition amongst Tomales Bay samples were driven by water-oyster differences while healthy and infected oysters remained comparable. This provides further evidence that the location of the host source location is a stronger driver of the oyster microbial community than the infection status. To further explore how site impact disease outcomes, future studies should include more sites, track environmental conditions (pH, temperature, salinity) and site characteristics (water flow, nutrient

availability, etc.), while tracking microbial dynamics along the development of the disease and explicitly testing microbial antagonisms like predation and competition.

Diversity metric analyses in my study illuminate the pivotal role of geographic location and habitat in shaping the diversity metrics for host-associated marine microbial communities. The presence of OsHV-1 did not significantly alter microbial diversity, suggesting a certain resilience or stability in the microbial community. Site-specific factors emerged as potential influencers, highlighting the importance of considering environmental parameters such as temperature, salinity, and nutrient availability as well as the potential pool of colonists when studying the effects of disease.

For both groups of samples, it is important to note that although there are fewer water samples than oyster samples, and thus the design is unbalanced, I am confident that the patterns observed are sufficiently strong to be meaningful. Particularly, a central result of this work is the strong effect of site on microbial community richness, Shannon Index, and microbial community composition.

Effects of OsHV-1 infection on Environmental Filtering

All oyster samples from both sites demonstrated sufficient filtering capabilities. Therefore, the infection status of Tomales Bay oysters does not hinder the oyster microbiome's ability to filter potential colonizers from their environment. If the virus were affecting the environmental filtering abilities of the oyster microbial community, one would expect the infected oyster microbiomes to closely resemble the microbial community of the environment, though this was not the case. Furthermore, the microbial

communities of Tomales Bay oysters are more similar to each other than to the environment. These similarities may be attributed to cohabitation with infected oysters (de Lorgeril et al., 2018; Pathirana et al., 2019; Lokmer and Wegner, 2015) or genetic traits of the host (Wegner et al., 2013).

Vibrio Abundance and Infection Relationship

In this strictly field approach, I report 6.0 genomic units of viral DNA per 1 µl of sample DNA while studies solely looking at viral load report $10^2 – 10^7$ (Pathirana et al., 2019; Paul-Pont et al., 2014). Furthermore, my study found an individual infection prevalence of 26% in contrast to a prevalence of 40 – 80% reported in previous laboratory based studies (Paul-Pont et al., 2013). This may be due to low expression in the field or a possible community resistance to the disease.

The study revealed a statistically insignificant correlation between *Vibrio* abundance and viral load. Field samples did not align with the patterns observed in controlled laboratory experiments (King et al., 2019; Petton et al., 2015; de Lorgeril et al., 2018), lacking a clear increase of *Vibrio* abundance in infected samples. Since OsHV-1 does not affect *Vibrio* abundances, I conclude that site-specific factors limit the increase of *Vibrio* abundance in the field, unlike what was observed laboratory studies (King et al., 2019; Petton et al., 2015; de Lorgeril et al., 2018).

The insignificant correlation between *Vibrio* abundance and viral load in field samples highlights the important role of specific environmental factors that are not variable in laboratory conditions (King et al., 2019; Petton et al., 2015; de Lorgeril et al.,

2018). These findings collectively emphasize the crucial role of natural habitats and their complexities in shaping disease dynamics, urging a holistic approach in studying and managing marine diseases.

Oyster Microbial Community Composition

Contrary to the initial hypothesis, pathogenic *Vibrio* did not predominate the microbial community composition in infected oysters; rather, they were only present in low quantities in healthy samples. Surprisingly, the community composition did not exhibit bacteria known to be etiological agents, and the microbial composition of infected oysters resembled that of healthy oysters at the same site. This finding suggests that bacterial composition varies with the environment (Prieur et al., 1990).

Delving deeper into the common genera enriches our comprehension of the biodiversity within the oyster microbiome, shedding light on potential functions. At the phylum level, the dominant taxon was Pseudomonadota (formerly Proteobacteria), consistent with previous work showing this clade as an inhabitant of oysters (Fernandez-Piquer et al., 2012; Pathirana et al., 2019). Across all sample types and at both sites, I consistently identified three shared genera: *Arcobacter*, *Halioglobus*, and NS5_marine_group. *Arcobacter,* a gram-negative spirillum bacterium, exhibits a wide habitat range and is a recognized constituent of the oyster microbial community (J. Fernandez-Piquer et al., 2012; Pathirana et al., 2019). Contrary to previous studies, my study provides little evidence that *Arcobacter'*s is involved in mortality events because it was highly abundant in both healthy Humboldt and Tomales samples (de Lorgeril et al.,

2018). *Halioglobus* is a gram-negative coccus bacterium that has been isolated from seawater (Dueholm et al., 2023). NS5_marine_group, or NS5, represents one of the most abundant marine flavobacteria in all the world's oceans, including arctic waters (Priest et. al. 2022). Like *Arcobacter*, *Halioglobus* and NS5 have not been studied in the context of disease systems, however, all bacterial genera are commonly found in marine systems, so their presence is not surprising.

There are two unique genera characterizing the microbial community of infected oysters from Tomales Bay: *Pleurocapsa PCC 7319* and *Polaribacter*. *Pleurocapsa PCC 7319* is a facultative photoheterotroph and has been previously isolated from marine snail shells (Dueholm et al., 2023). This strain of *Pleurocapsa* requires an elevated level of salts and are loosely defined cyanobacteria (Dueholm et al., 2023). *Pleurocapsa* has previously been associated with infections in *Mazzella laminarioides* (Corren et al., 1997) and is known to cause the development of tumors on fronds (Correa et al., 2000). Bacteria from the genus *Polaribacter* are capable of decomposing algae and are becoming more prevalent with the increase in algal blooms (Avcı et al., 2020). But there is no substantial evidence of a direct relationship between the presence of *Pleurocapsa* and/or *Polaribacter* and POMS.

CONCLUSIONS

My study shows that disease behaves differently in the field than would be expected based solely on previous lab experiments. Furthermore, while some studies propose that analyzing *Vibrio* abundance could serve as an indicator of oyster health (Pathirana et al., 2019), my results contradict this notion by revealing low *Vibrio* abundances in Tomales Bay oysters that still carried OsHV-1. Similarly, studies advocate for examining the microbial diversity of infected oysters as a strong health indicator (Lokmer and Wegner, 2015); however, my research revealed the opposite to be true.

Microbial community composition is influenced more by site than by infection status, a trend particularly evident in Tomales Bay oyster samples. Although laboratory experiments are of immense importance for understanding fundamentals of a system, the accuracy of these investigations could be compromised if they neglect to consider site-specific dynamics and the broader host-associated microbial community. Through a comprehensive understanding of site-specific factors, we can unravel the complex relationship between the oyster microbiome, OsHV-1 and the oyster environment, paving the way for enhanced disease management strategies and sustainable oyster health.

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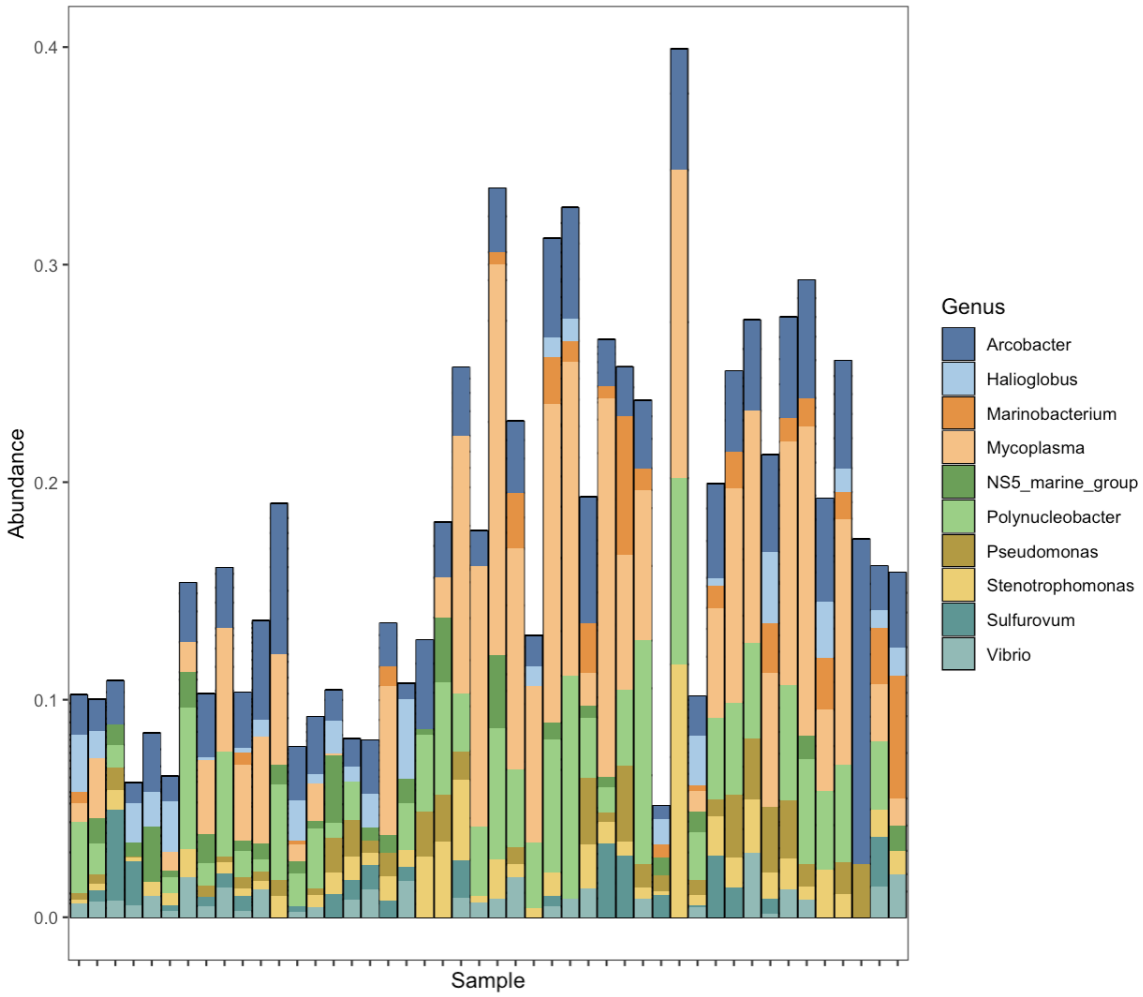
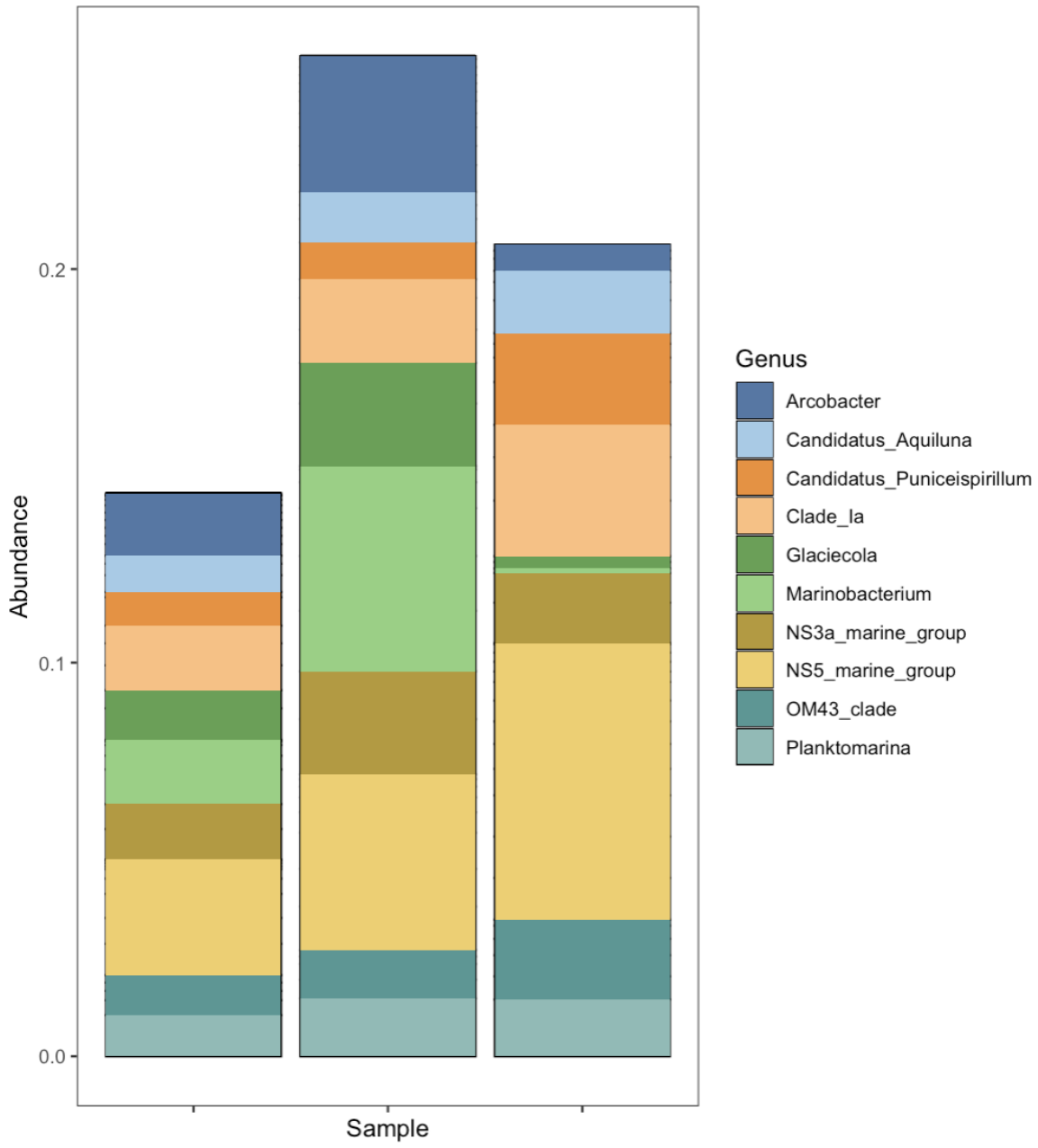
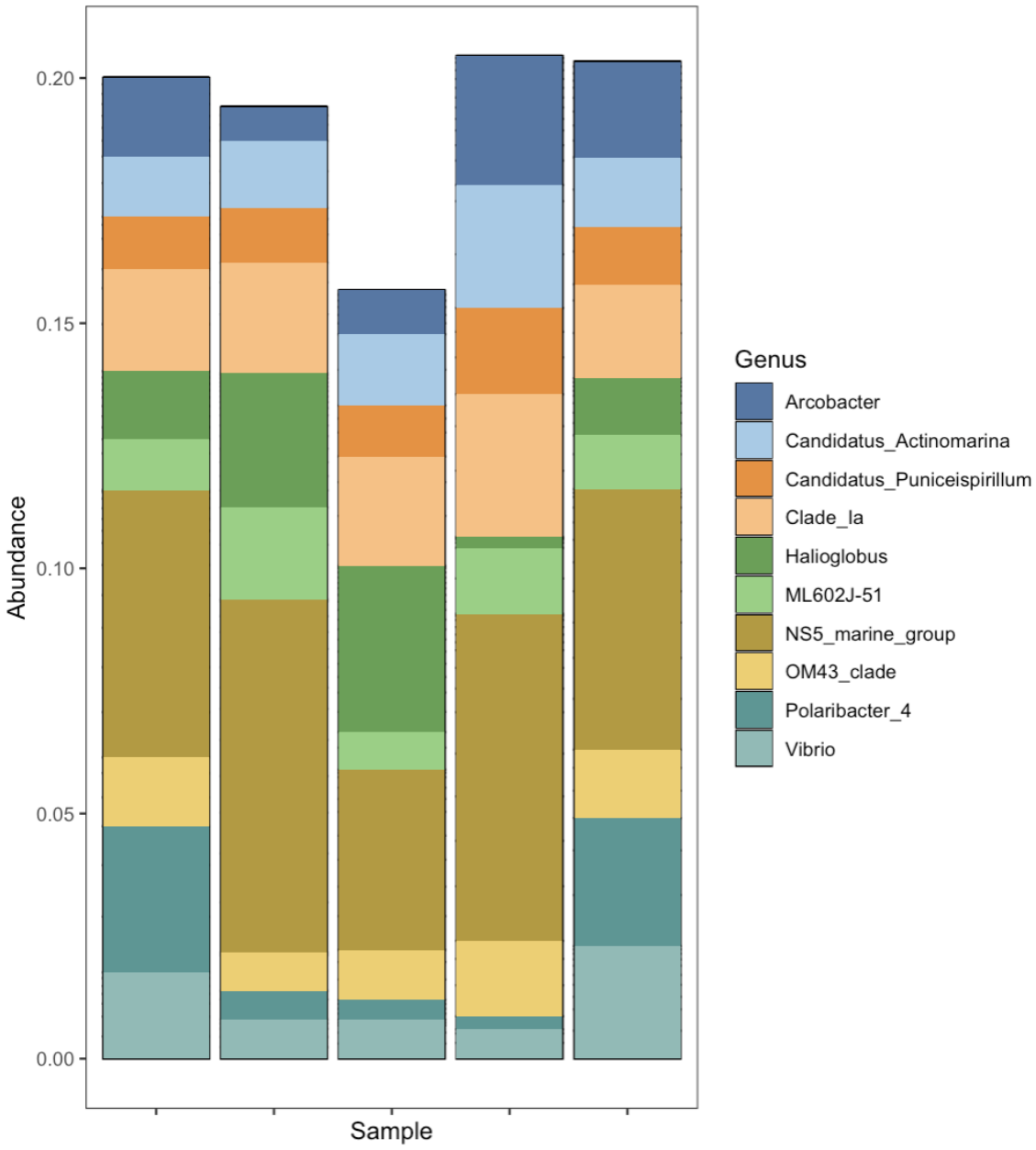
APPENDIX



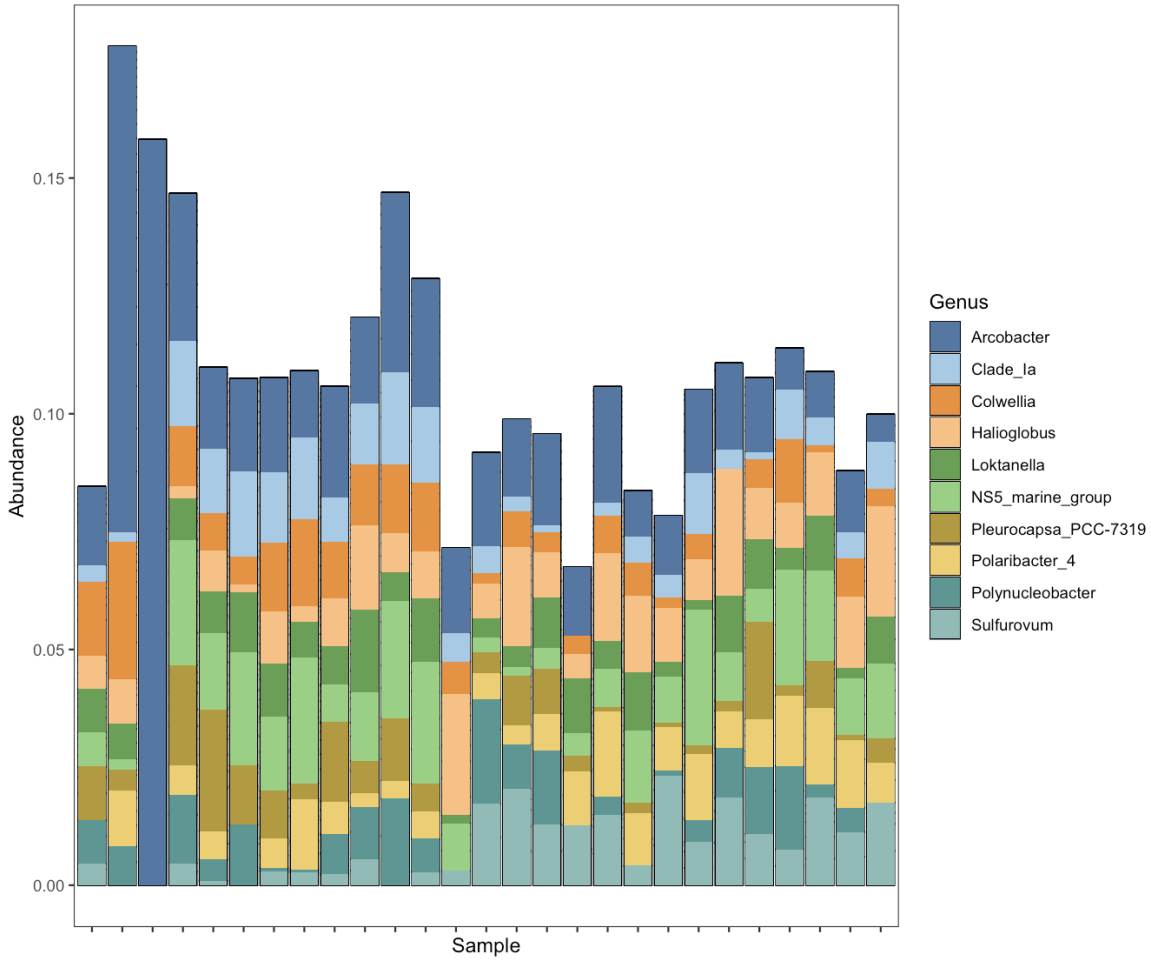
Appendix Figure 1. Results of 16S sequencing. Relative abundance of 10 most abundant genera of healthy Humboldt Bay oyster samples.



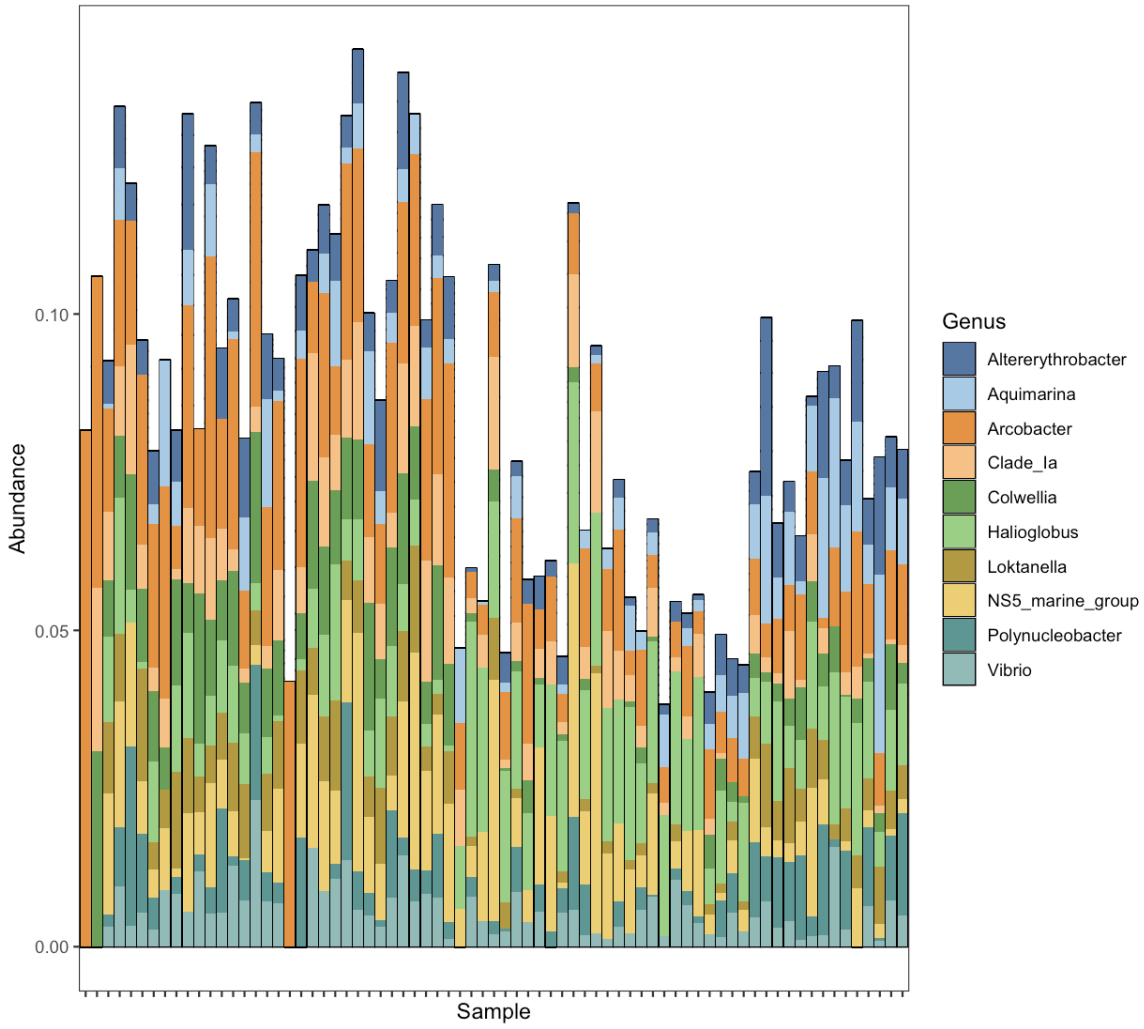
Appendix Figure 2. Results of 16S sequencing. Relative abundance of 10 most abundant genera of Humboldt Bay water samples.



Appendix Figure 3. Results of 16S sequencing. Relative abundance of 10 most abundant genera of Tomales Bay water samples.



Appendix Figure 4. Results of 16S sequencing. Relative abundance of 10 most abundant genera of infected Tomales Bay oyster samples.



Appendix Figure 5. Results of 16S sequencing. Relative abundance of 10 most abundant genera of healthy Tomales Bay oyster samples.