

Title: Temperature and microbe mediated impacts of the San Diego Bay ostreid herpesvirus (OsHV-1) microvariant on juvenile Pacific oysters

Running title: OsHV-1 San Diego Bay: thermal range and microbial response

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Abstract:

The ostreid herpesvirus (OsHV-1) was recently detected in San Diego Bay for the first time in farmed juvenile Pacific oysters (*Crassostrea gigas*). Due to the virus' ability to cause mass mortality (50 to 100%), it is important to determine the factors that promote infection as well as the consequences of infection. Here we assess the role of temperature in controlling OsHV-1 induced mortality. Pacific oysters were exposed to the San Diego Bay microvariant of OsHV-1 at 4 different temperatures (15, 18, 21, and 24°C). While OsHV-1 was able to replicate in oyster tissues at all temperatures, it did not induce mortality at 15°C, only at the higher temperatures. Additionally, we examined oyster tissue-associated bacterial response to OsHV-1 infection. As shown previously, bacterial richness increased following OsHV-1 exposure, and then decreased as the oysters became sick and died. Four bacterial taxa linked to the San Diego Bay microvariant infection, including *Arcobacter*, *Vibrio*, *Amphritea*, and *Pseudoalteromonas*, were the same as those shown for other microvariant infections in other studies from globally distributed oysters, suggesting a similar spectrum of co-infection irrespective of geography and microvariant type. The significant shift in the bacterial community following exposure suggests a weakening of the host defenses as a result of OsHV-1 infection, which potentially leads to adverse opportunistic bacterial infection.

Sustainability Statement:

The temperature threshold of OsHV-1 microvariant from San Diego Bay is critical information for management of the virus. With global ocean temperatures rising, the likelihood of OsHV-1-induced mortality outbreaks may increase. Microbial sequencing also further elucidated the presence of genus-specific bacterial activity in association with OsHV-1, which may be critical in disease progression and potential targets for combatting oyster disease. The impacts that OsHV-1 has on oyster aquaculture affect both food security (UN Sustainable Development Goal 2) and ocean sustainability (UN Sustainable Development Goal 14) because growth of aquaculture is essential to address seafood demand but disease outbreaks can cause negative consequences to both food supply and the health of the environment around those outbreaks.

Introduction:

The Ostreid herpesvirus (OsHV-1) is a pathogen that plagues aquaculture industries around the world, namely in France, Italy, Ireland, New Zealand, Australia and the West Coast of the United States (Burge *et al.* 2006, 2021, Segarra *et al.* 2010, Lynch *et al.* 2012, Martenot *et al.* 2012, Peeler *et al.* 2012, Jenkins *et al.* 2013, Keeling *et al.* 2014, Burioli *et al.* 2016, 2017, Abbadi *et al.* 2018). Microvariants of OsHV-1 (μ Var) which carry sequence variations from the reference genome (Davison *et al.* 2005) have evolved. They are highly virulent and lead to mass mortality events on oyster farms (Jenkins *et al.* 2013, Keeling *et al.* 2014, Burge *et al.* 2021, Mazaleyrat *et al.* 2022). Herpesvirus particles are seen in microscopy across various tissues, including adductor muscle, mantle and digestive gland, and specifically target oyster immune cells called hemocytes which may act as vehicles transporting the viral particles to different tissues (Schikorski *et al.* 2011, Martenot *et al.* 2017, de Lorgeril, Lucasson, *et al.* 2018). The virus attaches to host cells to invade them and replicate within (Martenot *et al.* 2019). Inhibition of apoptosis is frequently found in susceptible oysters and may be caused by viral reprogramming in favor of its own proliferation (Green *et al.* 2016, Martenot *et al.* 2017, de Lorgeril, Lucasson, *et al.* 2018, Picot *et al.* 2022). OsHV-1 may be detected and quantified by both DNA copies and mRNA expression levels, with quantitative PCR (Martenot *et al.* 2010, Burge and Friedman 2012, Agnew *et al.* 2020, Burge *et al.* 2020, 2021). Controlled laboratory studies have shown that OsHV-1 may be transmitted by filtered viral homogenate into naïve oysters through intramuscular injection, bath exposure with tissue homogenates or bath exposure using shed virus (Schikorski *et al.* 2011, de Lorgeril, Lucasson, *et al.* 2018, Burge *et al.* 2020). The virus is also spread through cohabitation, suggesting inter-oyster transmission on farms and in natural populations during outbreaks (Petton *et al.* 2013, de Lorgeril, Lucasson, *et al.* 2018).

OsHV-1 virulence is exacerbated by increasing temperatures. In fact, certain microvariants of OsHV-1 are incapable of inducing mortality below certain temperatures. An Australian microvariant was only found to induce mortality above 14°C (de Kantzow *et al.* 2016). Other microvariants, such as the French microvariant, have greatly reduced replication and almost no oyster mortality at lower temperatures such as 13°C (Pernet *et al.* 2015). As temperatures are increased, OsHV-1 induced mortality can increase up to 57% (μ Var, Delisle *et al.* 2018), 84% (μ Var, de Kantzow *et al.* 2016), 89.8% (μ Var, Petton *et al.* 2013) or even 100% (Burge *et al.*

2007). The most optimal range for most OsHV-1 microvariants appears to be between 16 and 26°C (Burge *et al.* 2007, Petton *et al.* 2013, Pernet *et al.* 2015, de Kantzow *et al.* 2016, Delisle *et al.* 2018, Pathirana *et al.* 2022). Optimal growth and function temperature for Pacific oysters is typically around 20°C (Wiltshire 2007). Higher temperatures may interfere with antiviral gene expression and cause differential response to OsHV-1, leading to higher likelihood of mortality (Green *et al.* 2014).

In San Diego Bay, temperatures vary from 15°C in winter to more than 22°C in summer (NOAA National Data Buoy Center:

https://www.ndbc.noaa.gov/view_climplot.php?station=sdbc1&meas=st). Temperatures reach as high as 25°C in San Diego Bay. The first detection of an OsHV-1 microvariant in San Diego Bay occurred in 2018 in farmed triploid juvenile Pacific oysters (*Crassostrea gigas*) (Burge *et al.* 2021). The detection of high OsHV-1 copy numbers in October of 2018 coincided with 99% spat mortality (Burge *et al.* 2021). This is a microvariant of OsHV-1 which is highly virulent, similar to French and Australian microvariants (Kachmar *et al.* in press). There have since been ongoing sentinel studies to monitor the spread of the OsHV-1 microvariant, which is more similar to the French, Australian and Chinese microvariants than to the Tomales Bay, California OsHV-1 variant (Burge *et al.* 2021). OsHV-1 San Diego Bay microvariant has since been detected during a mass mortality of small juvenile oysters in San Diego Bay in 2020 (Evans *et al.* submitted). The mitigation of this virus can be successful with selective breeding programs, informed planting times to avoid vulnerable periods based on age, temperature and other environmental factors, appropriate stocking density and limiting oyster transfer from one location to another (Dégremont *et al.* 2015, Burge *et al.* 2017, 2020, Divilov *et al.* 2019, 2021, Rodgers *et al.* 2019). Currently, a single oyster nursery exists in San Diego Bay, and the current management strategy is to allow commercial production of oysters only in times when the temperature is <20 °C, while sentinel oysters remain for early detection of outbreaks.

In addition to temperature, microbes may also play an important role in OsHV-1 infection. The ability of OsHV-1 to impair host immune defense facilitates opportunistic microbiota to invade and colonize the oyster. Oysters launch a predominantly antibacterial response at later stages of infection, hinting at the likelihood of secondary bacterial infections associated with viral infection (Green *et al.* 2016). This is further demonstrated by drastic shifts in microbiome community composition and bacterial accumulation with associated tissue damage upon histological examination of infected oysters (de Lorgeril, Lucasson, *et al.* 2018). A specific group of opportunistic bacteria is conserved across many OsHV-1 infectious environments, including *Arcobacter*, *Pseudoalteromonas*, *Amphritea*, *Marinomonas*, and *Marinobacterium* (de Lorgeril, Lucasson, *et al.* 2018, Delisle *et al.* 2022, Pathirana *et al.* 2022, Clerissi *et al.* 2023). It is also likely that *Vibrio* species contribute to disease and further its progression after initial infection with OsHV-1 (de Lorgeril, Lucasson, *et al.* 2018, Petton *et al.* 2019, Clerissi *et al.* 2020, 2023, Oyanedel *et al.* 2023). Some bacteria resulted in co-infections, while others may take advantage of both the host's immunocompromised state and resources produced by other bacteria, such as siderophores (Oyanedel *et al.* 2023).

As a result of the limited known detections in San Diego Bay, it is difficult to narrow down an optimal temperature range for the OsHV-1 San Diego Bay microvariant. Therefore, the first goal of this study is to assess the temperature threshold of this microvariant in naïve Pacific oysters without prior exposure to OsHV-1. The second goal was to explore microbial community dynamics after OsHV-1 exposure and assess the impact of bacteria on disease progression. This study has critical implications for OsHV-1 management in San Diego Bay, revealing a tight linkage between temperature and mortality, as well as demonstrating the ability for opportunistic and potentially detrimental bacteria to emerge during viral outbreaks.

Methods:

Oyster Infection Trials:

Due to the contagious nature of the virus, the experimental room used for infection was an enclosed, climate-controlled room with no flow-through seawater. Waterproof lab boots were worn at all times when in the room and a 10% bleach bath was prepared for washing the boots after working in the room. Lab coats and boots worn inside the room did not leave the area and were reserved strictly for use in this room. All trash that was accumulated from the experiment was disposed of in double-bagged biohazardous trash bags for off-campus autoclaving. All sharps, including needles and scalpels, were disposed of in biohazardous sharps containers. Drop cloths were placed over tanks to control possible aerosolization of the virus, therefore keeping a barrier between controls and OsHV-1 as a measure of contamination prevention. At the end of the experiment, all containers and materials which were in contact with oysters or OsHV-1 were soaked in 10% bleach for 30 minutes or sprayed with 10% bleach before rinsing and drying. All protocols were reviewed by the state of California and the facility inspected prior to the start of experiments.

Oyster Infection Experiment #1 - Mortality:

To address the first goal of the study, juvenile Pacific oysters were infected with OsHV-1 through bath exposure in experimental aquaria under four different temperatures (15, 18, 21, and 24°C) and monitoring mortality for 16 days after exposure.

Juvenile oysters (either 9 mm or 24 mm in length) were shipped on ice from an oyster farm in Humboldt Bay, California to Scripps Institution of Oceanography. Oysters had only been exposed to Humboldt Bay water, where OsHV-1 has never been detected (Burge CA, unpublished data, Elston RA unpublished data). All seawater used was from the Scripps Institution of Oceanography pier inlet, was at 35 ppt and was 0.2 micron-filtered prior to use. Immediately upon arrival, oysters were rinsed briefly with freshwater to clean off sediment and placed into tanks at 15 or 17°C. Oysters were split into either “donor” or “recipient” tanks. Water infected with OsHV-1 would later be transferred from donor oyster to recipient oyster tanks. The two donor tanks were 39 L Sterilite containers (Sterilite Corporation, USA) with 100 oysters (24 mm length) each starting at 17°C. Recipient tanks were split into two sides: OsHV-1 exposure or Control (Figure 1). Each side had four water baths to allow for the final acclimation of oysters to

four different temperatures (Figure 1). The recipient tanks were 1 L lidded containers with a hole in the lid for an airline. 10 oysters (9mm length) were placed in each of these recipient tanks and four tanks were placed into each water bath (Figure 1). One water bath on each side (both Control and OsHV-1 exposed) was kept at room temperature, which was 15°C, while all other water baths were started at 17 °C by using water heaters. Starting one day after the arrival of the oysters, the temperature in the water baths and the donor tanks was increased 1°C per day until the water reached the desired temperature. Donor tanks were increased to 21°C, while recipient tanks were made to be 15, 18, 21 and 24°C (Figure 1). Temperature loggers were placed in the recipient tank water baths at 21°C to monitor true water temperature fluctuation.

Water in every tank was changed completely every 2 days. All seawater used in the experiment was bleached at 10% before being disposed of down the sink drain. Oysters were fed *ad libitum* every day during the acclimation period. A mix of algae containing 50% *Chaetoceros muelleri*, 5% *Tetraselmis* sp., 20% *Tisochrysis lutea* and 25% *Nannochloropsis oculata* was boiled at 100°C for 1 minute to kill exogenous bacteria before being fed to oysters.

Once all tanks had reached their target water temperatures (21°C for donor oysters or 15, 18, 21 and 24°C for recipient oysters), the donor oysters were placed in a MgCl₂ bath (50 g/L) overnight to relax their adductor muscle. These donor oysters were injected with 100 µL of OsHV-1 homogenate with a total of 1x10⁶ viral copies using a 27-gauge needle injected directly into the adductor muscle. The homogenate used was a secondary pass from the original homogenate (Burge et al. 2021) prepared previously and cryopreserved at -80°C (Kachmar et al. in press). After waiting for 10 minutes, oysters were placed back into the 39 L containers with filtered seawater at 21°C for 48 hours. After 48 hours, a water sample was collected from each donor tank, extracted using the Zymo Quick-DNA Miniprep Plus Kit following the Biological Fluids and Cells Protocol and the concentration of OsHV-1 copies was quantified using qPCR (see full description below). The quantification of virus in the water in each tank was deemed sufficient for exposure (>1x10⁵ copies/mL). The water in the two donor tanks was mixed and another sample was taken to determine the final viral concentration in water (4.05x10⁶ copies/mL). 500 mL of the seawater carrying the virus was added to each recipient tank for every temperature on the OsHV-1 exposure side, dosing each set of 10 oysters with 2.03x10⁹ copies of OsHV-1. For oysters on the control side, tank water was changed with just filtered seawater.

To assess the magnitude of bacterial impact on OsHV-1 disease, an antibiotic treatment was applied to half of the tanks in all temperatures. For each temperature and condition (OsHV-1 exposed or control), there were four tanks with 10 oysters each (n = 32 tanks, n = 320 oysters). Two replicate tanks received antibiotics while the other two replicates did not (Figure 1). Starting with the first day of viral exposure and continuing at each water change, chloramphenicol was added at a final concentration of 10 mg/L as described in (Coffin *et al.* 2021), Ampicillin at a final concentration of 1 mg/L, and streptomycin at a final concentration of 0.1 mg/mL (Green *et al.* 2019) were added to the seawater immediately before adding the seawater to the antibiotic-treated tanks. Control tank water did not receive any additives. A follow-up experiment was conducted to validate the lack of difference in mortality between antibiotic and non-antibiotic treated oysters at 18°C. All conditions were replicated, except 20 oysters were used per

antibiotic treatment in an OsHV-1 exposure condition, and 10 oysters were used per treatment in the control tanks.

Oysters from all conditions were fed boiled algae immediately after virus exposure and 24 hours after virus exposure to ensure filtering of the virus but were starved for the remainder of the experiment. Oysters were monitored daily for mortality. Starting with the control side, tanks were lifted from the water bath, tapped to confirm that live oysters would close, and investigated further if any oysters remained gaping open. Any oysters that remained open were touched lightly with a pair of tweezers. If oysters did not close after this, they were deemed dead and removed from the tank. The tissue of the dead oysters was immediately removed from the shell, weighed, and stored in DNA/RNA shield solution at -20°C (Zymo Research). Once mortality leveled out, with less than 3 oyster deaths per day for 4 days, the experiment was terminated and tissue from all remaining live oysters was dissected and preserved in DNA/RNA shield solution.

Oyster tissue was homogenized in the DNA/RNA shield solution for 30 seconds using the soft tissue tips with the Omni Tissue Homogenizer tool (Omni International). Then, 500 µL of homogenate was used for DNA extraction using the Zymo Quick-DNA Miniprep Plus Kit following the Solid Tissues Protocol. This protocol involves a step of protein digestion for one hour. The extracted DNA template was stored at -20°C until use for quantitative PCR.

Oyster Infection Experiment #2 - Microbiome:

To address the second goal of this study, another bath exposure was performed at 21°C and oysters were visually assessed and categorized into different disease states (alive, sick or dead). The microbial patterns in community diversity across disease states and key bacterial players after exposure were compared with other studies to reinforce the theory that a common group of OsHV-1-associated bacteria are conserved across both geographic regions and across different microvariant infections.

Oysters from the nursery in Humboldt Bay were shipped to the climate-controlled room and placed in filtered seawater at 17°C. The temperature of the seawater increased 1°C/day until it reached 21°C. Water was changed every 2 days and oysters were fed boiled algae. Once oysters reached 21°C, 10 oysters were dissected, weighed and stored in DNA/RNA shield solution. Then, over 100 donor oysters were relaxed with an MgCl₂ salt bath overnight and were injected with 100 µL of OsHV-1 homogenate carrying 1x10⁶ total copies of OsHV-1 homogenate. After 48 hours, a water sample from the tank was collected, extracted and run through qPCR. The water contained 1.41x10⁶ copies/mL of OsHV-1. 500 mL of this virus water was added to each of 6 recipient tanks with 10 oysters per tank. After 4 days, 10 dead oysters and 2 sick oysters were collected from the tanks overall. Dead oysters failed to close after touching them with sterile tweezers, while sick oysters closed slowly and only after the tweezers touched their shells directly. Live oysters closed immediately after tapping the outside of their

tank. After 5 days, 8 more sick oysters and 10 live oysters were collected from the tanks overall and the experiment was terminated.

Oyster tissue was homogenized in the DNA/RNA shield solution for 30 seconds using the soft tissue tips with the Omni Tissue Homogenizer tool and stored at -20°C. Then, 500 µL of homogenate was sent for DNA extraction at the UCSD Microbiome Core using the MagMax Ultra Nucleic Acid Isolation Kit (Applied Biosystems). A portion of extracted DNA template was returned from the Microbiome Core and viral quantification was performed via qPCR.

qPCR:

Tank water and all oyster tissue samples were run through a qPCR reaction to determine if OsHV-1 was present and at what concentration. Methods were adapted from (Agnew *et al.* 2020, Burge *et al.* 2020, 2021). Each qPCR reaction well was prepared with 10 µL of Brilliant III Ultra-Fast SYBR QPCR master mix (Agilent), 0.8 µL of each 10 µM OsHV-1 primer (ORF100 Forward and Reverse (Burge and Friedman 2012, Burge *et al.* 2021)), 0.5 µL of Bovine serum albumin (BSA), 5.9 µL of molecular grade sterile H₂O and 2 µL of DNA template. All samples were run in triplicates. DNA standards, DNA negative extracts and qPCR controls were included in every run. The standard curve was prepared using a synthetic gBlock strand of the OsHV-1 ORF100 region (TGATGGATTGTTGGACGAGAGACAACAAGATAGTGCTGAGGATATGAAGAAAAAGGCC CAATGTATATAGACCCATGGCCGAAGCGAGAAACATATTTACTTTACAGTCTAATTTTGTAG CGTCCAGGGATGTGAT) in a dilution series from 2x10⁷ copies down to 20 copies/µL. Thermocycler (Eppendorf) conditions included one cycle of 20 seconds at 95°C, followed by 40 cycles of 3 seconds at 95 °C and 30 seconds at 60°C, and finished with a melt curve profile of 95°C for 15 seconds, 60°C for 15 seconds and a ramp up to 95°C held for 15 seconds. OsHV-1 copy number in unknown oyster and water samples was derived from the standard curve equation for that plate and averaged across the three replicates. Specificity of the amplified template was confirmed by comparing the melt curve of samples to that of the gBlock positive controls.

Microbiome Sequencing:

Control (n=10), live (n=10), sick (n=10) and dead (n=10) oyster microbial community libraries were prepared and sequenced by the UCSD Microbiome Core and the Institute for Genomic Medicine at UCSD. Library preparation was conducted using the KAPA Hyper Plus Kit (Roche Diagnostics, USA). The protocol for library preparation and sequencing is based on the Earth Microbiome Project, which uses primers 515F/806R to target the V4 region of the 16S rRNA gene in bacteria (Caporaso *et al.* 2012, Apprill *et al.* 2015). Sequencing by synthesis was conducted by the UCSD IGM Genomics Center on the Illumina MiSeq platform with paired-end 250 base pair cycles.

Analysis

Oyster Infection Experiment #1 - Mortality:

During the 16-day period following OsHV-1 exposure, oysters were monitored every day for mortality. If an oyster was found dead, it was recorded as a “1”, along with its temperature, exposure condition (control vs. OsHV-1 Exposed), antibiotic treatment (yes or no), and the days since exposure for when it was found dead. Each mortality was recorded as an individual data point. At the end of the experiment, any remaining live oysters across all variables were recorded individually as “0”. The data on mortality was tracked in this way to perform a survival analysis using the Kaplan-Meier estimate (Kaplan and Meier 1958). In R, the Kaplan Meier survival estimate was computed with the function *survfit()* from the package *survival* (Therneau and Grambsch 2000, Therneau 2023). Then, the step function plots were created using *ggsurvplot()* from the *survminer* package. Finally, a log rank test was conducted with the *survdif()* function in the *survival* package to determine whether mortality varied significantly across conditions.

Whole oyster tissue was collected from this experiment and all tissue was assessed for OsHV-1 viral load. Quantitative PCR data for all OsHV-1 exposed oysters was normalized by weight of tissue in grams, because samples were collected in a standardized volume. The log of the normalized copy numbers by temperature and mortality status (live vs. dead) were visualized with violin plots. Kruskal Wallis tests were used to determine whether OsHV-1 copy number varied between live and dead oysters and across temperatures.

Oyster Infection Experiment #2 - Microbiome:

Sequencing reads from the control, alive, sick and dead oyster samples were demultiplexed with QIIME2 (Bolyen *et al.* 2019). DADA2 from Qiime2 v2020.6 was used to denoise the data to produce a feature abundance table of Amplicon Sequence Variants (Callahan *et al.* 2016). Reads were truncated at 224 base pairs on the forward reads and 200 base pairs on the reverse reads based on sequence quality plots. Taxonomic classification of reads was conducted with the Qiime2 pre-trained classifier from Greengenes2 v2022.10 (McDonald *et al.* 2023). ‘Unassigned’ taxa were filtered out of the feature abundance table. A phylogenetic tree was constructed using the SEPP fragment insertion method (Janssen *et al.* 2018). The DADA2 ASV abundance table, Greengenes2 taxonomy file, and phylogenetic tree were imported into R as a phyloseq object. Phyloseq v1.42.0 was used to calculate Richness, Evenness, Shannon’s, and Simpson’s alpha diversity (McMurdie and Holmes 2013). Kruskal Wallis tests with pairwise Dunn’s test were used to determine statistical significance between groups. An alpha correlation test was run to determine strength of linear correlation between evenness and viral load. Richness values were plotted against evenness values as proposed by (Gauthier and Derome 2021). A distance matrix was calculated with unweighted UniFrac using the Phyloseq package and plotted with Principal Coordinates Analysis (Lozupone *et al.* 2006, McDonald *et al.* 2018). Adonis tests were run on unweighted UniFrac distances to determine significance of both

disease state and viral load in altering microbial composition. Hierarchical clustering was conducted with the unweighted UniFrac distance matrix and hclust using the complete linkage method. The tree was cut into its primary 5 clusters. Differential abundance analysis was conducted with ANCOM-BC, which corrects the bias from uneven sampling fractions across samples, uses a log-linear regression, and conducts multiple pairwise comparisons all while controlling the multi-direction false discovery rate (Lin and Peddada 2020). ANCOM-BC is robust to the sparse matrices used in microbiome data and adaptive to structural versus sampling zeros. The disease state of the oyster sample was used as the fixed formula for ANCOM-BC with an initial prevalence cutoff of 10% and Holm p-value adjustment. Pairwise comparisons were conducted against the control samples (before OsHV-1 exposure) as the reference, which produced log fold change values from control to alive, control to sick, and control to dead oyster samples. An adjusted p-value of less than 0.05 was used as the cutoff for significantly different taxa, which were agglomerated at the genus level. Finally, Random Forest analysis with *randomForest()* (Liaw and Wiener 2002) package and function in R was performed to assess the ability to predict disease state and OsHV-1 exposure status based on the microbial composition and abundance data. A prevalence cutoff of 10% for each sample set was used again to remove rare taxa. Out of bag error rate was calculated for two comparisons: Alive versus Dead oysters and Control (“Before”) vs. Alive & Sick (“After”) oysters. The top 20 ASVs that were most important for predicting condition in either comparison were selected based on the greatest mean decrease in GINI coefficient, which equates to the ASVs improving predictive capabilities at a given node in the decision tree.

Results:

Oyster Infection Experiment #1 - Mortality:

Mortality varied significantly by temperature but did not vary with antibiotic treatment. No oysters in the control group, at any temperature with or without antibiotic treatment died, thus only OsHV-1 exposed oysters were plotted for mortality. At 15°C, no oysters died in any of the temperatures either with or without antibiotic treatment (Figure 2). In all other temperatures, there was no significant difference in mortality between antibiotic treated or untreated oysters (Figure 2). Log rank tests were used to determine whether mortality was significantly different between temperatures. Overall, mortality did vary significantly between temperatures ($p < 0.0001^*$) (Figure 2). Mortality at 18°C was significantly different from that of 15°C ($p < 0.0001^*$), 21°C ($p = 0.00065^*$) and 24°C ($p = 0.00227^*$). However, mortality at 21°C did not vary from 24°C ($p = 0.66852$). Over 75% of oysters died in all temperatures 18°C and higher, but oysters at 21°C and 24°C started dying sooner after OsHV-1 exposure than oysters at 18°C (Figure 2). At 18°C, antibiotic treated oysters appear to start dying later than untreated oysters, but this difference was not statistically significant in either the initial or the follow-up experiment (Figure 2).

OsHV-1 load was much higher in dead oysters compared to remaining live oysters but did not vary by temperature. Kruskal Wallis tests were conducted separately for live and dead oysters. Within the dead oysters, OsHV-1 normalized qPCR values did not significantly differ between

temperatures ($p = 0.168$) (Figure 3). Similarly, OsHV-1 load did not significantly differ between temperatures within remaining live oysters ($p = 0.7112$) (Figure 3). A Wilcoxon test (or Mann Whitney test) was conducted to determine whether OsHV-1 load varied between dead and live oysters across all temperatures combined. OsHV-1 load was significantly higher in dead oysters compared to live oysters ($p < 0.001^*$) (Figure 3). The variability of normalized qPCR values was much higher in remaining alive oysters, while values for dead oysters were all very similar (Figure 3).

Oyster Infection Experiment #2 - Microbiome:

Following infection, the tissue-associated microbial alpha diversity decreased over the spectrum of mortality (alive>sick>dead; Figure 4). Figure 4 demonstrates two alpha diversity measures across oyster disease states (Shannon's and Simpson's) and then breaks down these metrics into their components by plotting Richness against Evenness. Richness and Evenness are both components of Shannon's and Simpson's index, with richness having more weight in Shannon's index and evenness having more weight in Simpson's index. After exposure to OsHV-1, but while oysters were still alive or sick, Shannon's and Simpson's overall diversity measures were unchanged (Kruskal Wallis(KW) & Dunn's test(D)/ $p > 0.05$)(Figure 4). However, Richness increased significantly from before exposure to after (Control – Alive/ KW&D/ $p = 0.0012^*$; Control – Sick/ KW&D/ $p = 0.0012^*$)(Figure 4). Evenness only started to decrease once oysters show signs of sickness (Control – Sick/ KW&D/ $p = 0.0026^*$)(Figure 4). Once oysters were found dead, Shannon's and Simpson's diversity decreased significantly (Figure 4). In dead oysters, Richness returned to a similar level to before OsHV-1 exposure (Control – Dead/ KW&D/ $p = 0.4515$) and was significantly lower than remaining sick and alive exposed oysters (Alive – Dead/ KW&D/ $p = 0.0086^*$; Sick – Dead/ KW&D/ $p = 0.0073^*$)(Figure 4). However, Evenness decreases in dead oysters and was significantly lower than oysters before exposure and oysters that were still alive without signs of sickness (Control – Dead/ KW&D/ $p = 0.001^*$; Alive – Dead/ KW&D/ $p = 0.0228^*$)(Figure 4). Evenness was also significantly negatively correlated with viral load. As OsHV-1 load (log(copies/g)) increased, evenness decreased (alpha correlation, $r = -0.7318$, $p < 0.0001^*$).

The microbial composition of oyster tissues changes after OsHV-1 exposure and shifts again once the oyster has died. Figure 5 demonstrates the clustering of similar samples based on their microbial communities. Unweighted UniFrac (beta diversity metric) looks at ASVs present but not their abundance. UniFrac also accounts for phylogenetic similarity based on the SEPP fragment insertion phylogenetic tree. Unweighted UniFrac shows significant difference between the microbial constituents of control oysters and alive oysters (adonis, $p = 0.006^*$), as well as control oysters and sick oysters (adonis, $p = 0.006^*$), but not between alive and sick oysters (adonis, $p = 0.906$) (Figure 5). Dead oysters also have a significantly different beta diversity compared to control, alive and sick oysters (adonis, all pairwise comparisons $p = 0.006^*$)(Figure 5). Unweighted and weighted Unifrac distances are also significantly associated with viral load (adonis, Unweighted: $R^2 = 0.064$, $p = 0.001^*$, Weighted: $R^2 = 0.224$, $p = 0.001^*$). Hierarchical clustering breaks the oyster samples into 5 distinct clusters (Figure 5). Three clusters are composed of only control oysters, and control oysters are not found in either of the other two

clusters (Figure 5). One cluster contains a mix of alive and sick oysters with a few dead oysters (Figure 5). The final cluster contains only dead oysters (Figure 5). The oysters which cluster into groups together are most likely to be found in close positions on the Unweighted UniFrac plot as well, suggesting some overlap in the ASVs found in these samples. Most notable is the clear separation between unexposed and OsHV-1-exposed oysters in terms of both Unweighted UniFrac ordination and Hierarchical clustering (Figure 5).

There were 27 genera found to be differentially relatively abundant after conducting pairwise comparisons between control oysters and alive, sick or dead oysters with ANCOM-BC. The heatmap in Figure 6 displays significant log fold change values (Holm-adjusted p value < 0.05) for each genus for each pairwise comparison. Only one genus was found to significantly decrease in abundance from the control group (Figure 6). The cyanobacteria Family Coleofasciculaceae was a significantly lower proportion of the community in the sick oysters (Figure 6). Many genera were more abundant in both alive and sick oysters compared to control oysters, but not in dead oysters (Figure 6). These include 2 genera from Verrucomicrobiales (one of which is from the family Akkermansiaceae), the Proteobacterial groups *Eionea* and Micavibrionaceae, a Planctomycetota, a Myxococcota group, and a Delongbacteria group (Figure 6). There were also groups that were more abundant in both sick and dead compare to control, but not alive and apparently healthy oysters (Figure 6). These are *Vibrio*, *Kiloniella* and Arcobacteraceae (Figure 6). No genera were found to be more abundant in both alive and dead, but not sick oysters (Figure 6). Two Planctomycetota groups (*GCA-2683825* and *Thalassoglobus*) were only more abundant in alive oysters post-OsHV-1 exposure. (Figure 6) Various groups were only more abundant in sick oysters, such as Arenicellales, Kapabacteriales, Flavobacteriaceae, and *Flavilitoribacter* (Figure 6). Four groups were only more abundant in dead oysters; an unknown genus of Nitrospirales, 2 Alteromonadaceae, one of which was *Pseudoalteromonas*, and *Phaeobacter* (Figure 6). Finally, various taxa were found to be more abundant in all oyster disease states after OsHV-1 exposure, including *Amphritea* (which increases in log fold change with increasing severity of disease state), *Sneathiella*, *Peredibacter*, Vicingaceae, and Crocinitomicaceae (Figure 6).

Of the genera found to be differentially abundant by ANCOM-BC, 9 were also identified to be predictive of disease state using Random Forest analysis (Figure 6, Table 1). These are both Verrucomicrobiales genera, *Eionea*, *Amphritea*, *Vibrio*, *Pseudoalteromonas*, Micavibrionaceae TMED27, *Kiloniella*, and *Flavilitoribacter* (Figure 6, Table 1). These taxa were most important based on the greatest mean decrease in the Gini coefficient, which means that these taxa increased the ability of the model to predict which group the sample was from. Random Forest models were run on two different subsets of the samples. One model was run on control oysters ($n = 9$) versus alive and sick oysters grouped together ($n = 20$), which was “Before versus After OsHV-1 Infection” (Table 1). Dead oysters were removed from this model because dead oyster tissue likely contains many bacteria involved in decomposition, but not directly associated with OsHV-1 infection. This model had an out of bag error rate of 3.45%. The second model was run on alive ($n = 10$) versus dead ($n = 10$) oysters to determine which bacteria were responsible for moving between disease states once the oyster was already exposed to the virus (Table 1). This model had an out of bag error rate of 10%. Some taxa were found to be important for

model prediction in both “Before versus After OsHV-1 infection” and “Alive versus Dead oysters” (Table 1). These include Akkermansiaceae, *Roseovarius*, and *Amphritea spongicola* (Table 1).

Taken together, ANCOM-BC and Random Forest results can be more robustly assessed. For example, Akkermansiaceae, *Flavilitoribacter*, *Vibrio*, *Kiloniella*, *Amphritea*, Verrucomicrobiales genus Arctic95d-9, *Eionea*, and Micavibrionaceae TMED27 were all found to be important for predicting whether oysters had been exposed to OsHV-1 or not (Table 1). In ANCOM-BC, these genera were also significantly different between exposed and unexposed oysters, with all groups having increased in the exposed oysters (Figure 6). In predicting whether oyster samples came from surviving or dead oysters under OsHV-1 exposure, *Pseudoalteromonas*, *Vibrio*, *Amphritea*, and Akkermansiaceae were all found to be important (Table 1). In differential abundance analysis, *Pseudoalteromonas*, *Vibrio* and *Amphritea* all increased as a proportion of the microbiome in dead oysters (Figure 6). Akkermansiaceae is significantly increased in surviving oysters, but not in dead oysters (Figure 6).

Discussion:

Oyster Infection Experiment #1 - Mortality:

Mortality in this experiment is expected to be due to the interaction between temperature and OsHV-1. Temperature alone was not responsible for killing oysters, because no control oysters died during experimentation. Marine diseases are tightly linked with temperature. In abalone, Withering Syndrome increases under elevated seawater temperatures (Moore *et al.* 2000) while abalone herpesvirus infections only occur at temperatures below 23 °C (Gu *et al.* 2019). Both diseases result in mass mortalities. The virus responsible for causing white spot syndrome in shrimp is also highly dependent on temperature, with one study finding that the optimal range for this virus to cause disease is between 23 and 28 °C (Guan *et al.* 2003). The link between increased water temperatures and disease has been especially well documented in corals, which have been monitored for disease for decades (Harvell *et al.* 1999, 2002, Burge *et al.* 2014). Exposure of pathogens to new hosts via range expansion of either the host or the pathogen is in part driven by changes in ocean heat content making new areas suitable for invasion (Harvell *et al.* 1999, Burge *et al.* 2014). This may be exacerbated by human-mitigated introduction of new species or populations (Harvell *et al.* 1999). Spread of Dermo disease and MSX in eastern oysters (*Crassostrea virginica*) on the East Coast of the United States was likely driven by warming ocean temperatures (Harvell *et al.* 1999, 2002, Burge *et al.* 2014). Temperature can also impact virulence of pathogens in their current ranges and alter host susceptibility through physiological stress (Harvell *et al.* 1999, 2002). There may also be overlap in the optimal growth temperature of known pathogens with other opportunistic bacteria, fungi and viruses which creates conditions for polymicrobial infections (Harvell *et al.* 2002).

Colder temperatures have previously been shown to decrease mortality in OsHV-1 exposed oysters (Petton *et al.* 2013, Pernet *et al.* 2015, de Kantzow *et al.* 2016). For the French microvariant, oyster survival is highest at 13°C or lower (Petton *et al.* 2013, Pernet *et al.* 2015). For the Australian microvariant, OsHV-1 was not capable of killing oysters when the

temperature was dropped to 14°C, and a threshold for productive viral infection and mortality is likely between 14°C and 18°C (de Kantzow *et al.* 2016). Low temperature likely permits more effective antiviral response by oysters hosts, allowing oysters to survive by limiting viral replication (Green *et al.* 2014). In the case of the San Diego Bay microvariant assessed in this study, temperatures of 15°C or lower are likely to confer 100% survival against OsHV-1 infection. While quantification of viral copies showed that the virus was able to replicate in at least some oysters at the low temperature, this did not lead to mortality. Therefore, in San Diego Bay, naïve juvenile oysters are unlikely to become lethally infected with OsHV-1 at these low temperatures.

As temperatures increase, the ability of OsHV-1 to infect oysters increases. In this experiment, the optimal temperature for the San Diego Bay OsHV-1 microvariant was 21 to 24°C. Oysters died faster at these temperatures than at 18°C, which may be due to increased metabolism at higher temperatures promoting increased viral replication (Petton *et al.* 2023). Mortality after just 2 days of bath exposure has been documented previously (Burge *et al.* 2020). However, overall survival was very low at temperatures above 18°C, which indicates that any temperature that is permissive to the virus creates a big risk for juvenile oysters. In other OsHV-1 variants and microvariants, mortality also increases with temperature (Friedman *et al.* 2005, Petton *et al.* 2013, Delisle *et al.* 2018). The Tomales Bay, CA variant causes outbreaks during the summer which are primarily associated with temperature extremes above 24°C (Friedman *et al.* 2005, Burge *et al.* 2006). The French OsHV-1 microvariant tends to be most virulent at temperatures between 16 and 26°C (Petton *et al.* 2013, Delisle *et al.* 2018). Above this temperature range, oyster mortality is decreased, potentially due to altered oyster physiology and more efficient immune response (Petton *et al.* 2013, Delisle *et al.* 2018, 2020). The highest temperature tested in this study was 24°C, but it is possible that higher temperatures could inactivate and limit replication of the San Diego Bay OsHV-1 microvariant. For the Australian OsHV-1 microvariant, mortality increases severely at 22 and 26°C, with 26°C likely being the optimal temperature for inducing mortality (de Kantzow *et al.* 2016). The San Diego microvariant likely has a similar temperature threshold to other OsHV-1 microvariants, but viral copies are still found in oysters at 15°C after 16 days, suggesting infection is still possible at these lower temperatures if the virus is introduced.

All dead oysters had very similar viral loads regardless of temperature, while remaining alive oysters had highly dispersed viral loads at all temperatures. These dispersed viral loads suggest that individual oysters were at various stages of infection. The viral load and likelihood of mortality also depend highly on genotype, as some families could be more resistant to OsHV-1 infection than others (de Lorgeril, Lucasson, *et al.* 2018). Burge and Friedman 2012 suggests that viral gene expression is highest in the first few days after infection (Burge and Friedman 2012). Infection may be occurring at different times for each individual oyster, as 10 oysters are housed together, and they may be feeding at different times and rates. Low viral load below 10⁶ copies/mg (Burge *et al.* 2021) suggests that the oyster was tolerant of the viral infection or was able to become gradually infected from bath exposure and from viral shedding of other oysters throughout the experiment. Higher viral loads in live oysters may be predictive of further mortality, had the oysters been monitored for longer. Oysters may be able to tolerate high viral

loads without succumbing to death, which was demonstrated by high viral loads (10^8 copies) in surviving oysters infected with the French and Australian microvariants (Agnew *et al.* 2020). Some viral loads in alive oysters overlapped with dead, further suggesting that surpassing a threshold of viral copies may not be the only predictor of death. Although many oysters exposed to OsHV-1 at low temp were able to clear the virus, another study showed that OsHV-1 load increased again when moved to warmer temperatures (Pernet *et al.* 2015). Once the virus is present in an oyster, it is likely latent and can persist until warmer temperatures are able to re-activate it (Petton *et al.* 2015). This is likely a part of the cause for reactivation during 2020 In San Diego. Outbreaks have only occurred in San Diego Bay in 2018 and 2020, despite temperatures rising well above 18°C every summer. Infected oysters were entirely removed from San Diego following OsHV-1 detection, but OsHV-1 may survive latently in wild oysters or other unknown sources (Burge *et al.* 2006). OsHV-1 has never been detected in wild oysters in San Diego Bay, but these oysters may be tolerant of the virus and keep viral copies below the limit of detection. Temperature is likely not the only reason for OsHV-1 activation, but additional factors leading to OsHV-1 dispersal are unknown.

Antibiotics had no effect on mortality rates in this experiment. Therefore, the OsHV-1 San Diego Bay microvariant is capable of killing oysters even with a suppressed bacterial community, which differs from other microvariants where *Vibrio* colonization is essential to full disease expression (Petton *et al.* 2015). As was used in a prior study, chloramphenicol was utilized as an antibiotic to limit bacterial colonization of oysters during OsHV-1 infection (de Lorgeril, Lucasson, *et al.* 2018). The study by de Lorgeril *et al.* found that antibiotic treatment significantly reduced mortality in OsHV-1 challenged oysters (de Lorgeril, Lucasson, *et al.* 2018). Based on gene expression analysis, OsHV-1 infection places oysters in an immunocompromised state, allowing pathogenic bacteria to overcome host immune defenses and leading to lethal bacterial infections (de Lorgeril, Lucasson, *et al.* 2018). However, the present study did not inoculate *Vibrio* pathogens into the seawater alongside OsHV-1, which would have ensured the presence of a bacterial pathogen with capacity to kill oysters. It is possible that the oysters used in this study did not contain any known pathogenic bacteria from the start of the experiment, which would have been necessary to see the difference between antibiotic treated versus untreated oysters. Alternatively, the viral infection may have been highly potent and efficient in dampening oyster immune response in the specific experimental setting used, whereas a weaker viral infection would have permitted a stronger effect of the antibiotics on survival. Therefore, antibiotics did not offer any additional survival benefit, even if they altered activity of opportunistic bacteria within the oyster microbiome. In other words, the OsHV-1 San Diego Bay microvariant can kill oysters independently of other infections under the right conditions. This experiment focused on juvenile stage oysters, but age has been found to play an important role in OsHV-1 susceptibility (Hick *et al.* 2018). As such, these results may not directly apply to other age groups, such as oyster seed or adults.

Oyster Infection Experiment #2 - Microbiome:

Bacterial community diversity deteriorates as OsHV-1 induced disease progresses to mortality. Microbial alpha diversity metrics, such as Shannon's and Simpson's indices, remain unchanged

following exposure of Pacific oyster spat to an OsHV-1 microvariant in New Zealand (Delisle *et al.* 2022), but these metrics do not portray specific changes in either richness or evenness, which can change in themselves without impacting Shannon's or Simpson's diversity. Microbe richness increases after OsHV-1 exposure but decreases as the oysters display illness symptoms, and then die. Based on previous studies, both absolute abundance of bacteria and number of different bacterial species commonly increase during OsHV-1 infection (de Lorgeril, Escoubas, *et al.* 2018, de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023). De Lorgeril *et al.* 2018 refers to this period as a destabilization of the bacterial community, which coincides with a decrease in antimicrobial products (de Lorgeril, Lucasson, *et al.* 2018). Oyster defenses against bacteria are likely suppressed by viral infection and this disturbance likely promotes the growth of bacteria which were previously susceptible to host immune responses. Once the oyster has died, there is no more host immune response to bacterial invaders and no control over the bacterial community within the tissue. This allows for the bacteria community to shift based on environmental controls and competition for resources rather than host mitigation. At this point, the community has likely shifted to a state of decomposition favoring oyster spoilage bacteria (Chen *et al.* 2019) and appears to select for dominance of fewer bacterial groups. This is further reflected by the paired drop in evenness when oysters become visibly sick. Bacteria previously promoted by the oyster immune system have likely lost protection with a deficient immune response. As the oysters immune system is weakened by viral proliferation, microbes are also able to proliferate (de Lorgeril, Escoubas, *et al.* 2018), but competition between bacteria leads to winners and losers in the oyster microbiome. Certain taxa outcompete others and grow in abundance, while many other taxa die out, leaving an uneven distribution of bacterial abundances. In other studies, evenness was found to predict oyster survival against OsHV-1, as resistant oyster families started off with higher bacterial evenness measurements and maintained high evenness during infection (Clerissi *et al.* 2020). Evenness is an important measure to consider with OsHV-1 infection because the imbalance of bacteria in susceptible oysters is likely an additional stress to the host. Taken together, the changes in richness and evenness are likely due to a weakening immune system brought on by viral infection, which leads to a re-assembling of the bacterial community and a transition towards an opportunist-dominated microbiome and eventually mortality.

Community composition is significantly impacted by OsHV-1 exposure. After OsHV-1 exposure, there is a significant shift towards a new community of bacteria. This community is highly similar between alive and sick oysters (Fig. 5). Multiple studies show a strong shift in the microbiome from before to after OsHV-1 exposure (Clerissi *et al.* 2020, Delisle *et al.* 2022), with the greatest change occurring after 24 hours of exposure (de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023). This change likely occurs alongside exponential viral replication and dysfunction of hemocytes (de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023). Although oysters were not sampled until at least 4 days after exposure in this study, the shift in the microbiome likely occurred after just 24 hours if the disease behaves similarly to prior studies (de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023). There is strong overlap between alive and sick oysters in hierarchical clustering analysis. Some of the dead oysters are also close in microbial composition to alive and sick oysters, one explanation for which is that they died more recently and are yet to decompose. Disease states were subjectively assigned based on behavior and

appearance, but microbial composition may do a better job at suggesting disease stage than visual appearance and could even provide an estimation of time since death (Metcalf *et al.* 2016). Another shift in composition happens once oysters die. Similar to alpha diversity, this likely results from a lack of host control over the microbial community, causing exclusion of certain bacteria and favoring decomposing bacteria. The timing of viral replication is likely the most important factor dictating microbial community composition, as demonstrated in previous studies (de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023), as opposed to visual characteristics of exposed oysters. Therefore, the taxa responsible for these shifts in composition may be closely linked to OsHV-1 replication and altered hemocyte function.

Certain bacterial taxa were significantly associated with the transition between disease states. The only bacteria group found to significantly decrease in any comparison was cyanobacterial family Coleofasciculaceae, which was far less represented in sick oysters compared to pre-exposure oysters. This family of cyanobacteria has not been reported in oyster tissue before, preventing a thorough assessment of whether the loss of the bacteria is a sign of declining oyster health. Taxa found in mostly live and sick oysters that were exposed to OsHV-1 may take advantage of the host's compromised state. In this study, these taxa include Akkermansiaceae, *Flavilitoribacter*, *Vibrio*, *Kiloniella*, *Amphritea*, Verrucomicrobiales genus Arctic95d-9, *Eionea*, and Micavibrionaceae TMED2. Each of these taxa were also important for predicting whether oysters fit into pre- or post-OsHV-1 exposure status. *Vibrio* and *Amphritea* specifically have been identified in association with OsHV-1 infected oysters across many studies (de Lorgeril, Lucasson, *et al.* 2018, Delisle *et al.* 2022, Pathirana *et al.* 2022, Clerissi *et al.* 2023) and increased in this study with increasing severity of disease state. Additionally, *Arcobacter* was found to be important for distinguishing between exposed and unexposed oyster samples, although the genus itself was not identified in the differential abundance analysis between disease states. Of all these taxa, *Arcobacter*, *Vibrio* and *Amphritea* were found to have significantly higher transcriptional activity than other bacteria during OsHV-1 infection in Clerissi *et al.* 2023. Some of these bacteria may be functionally complementary and take advantage of the weakened host state (Clerissi *et al.* 2023). On the other hand, certain *Vibrio* species are known to act synergistically with OsHV-1 to accelerate the disease, working in tandem to cause hemocyte damage and impair immune defenses (Oyanedel *et al.* 2023). *Peredibacter* is another bacteria overrepresented in exposed oysters of all states which has been seen with OsHV-1 infection before but only early on in infection (Clerissi *et al.* 2023). Other bacteria in this study were found to be primarily associated with mortality or predictive of whether oysters were alive or dead, such as *Pseudoalteromonas*, *Phaeobacter* and Cryomorphaceae. *Pseudoalteromonas* and *Phaeobacter* were also found to be associated with OsHV-1 in Clerissi *et al.* 2023, but only *Pseudoalteromonas* demonstrated high transcriptomic activity during infection (Clerissi *et al.* 2023). Cryomorphaceae was overrepresented in OsHV-1 infected oysters in both Clerissi *et al.* 2023 and de Lorgeril 2018 (de Lorgeril, Lucasson, *et al.* 2018, Clerissi *et al.* 2023). In this study, *Pseudoalteromonas* and *Phaeobacter* are more unique to dead oyster samples, which may either suggest that they have an important role in the disease progression, alongside *Arcobacter*, *Vibrio* and *Amphritea*, or they are just coincidental with dying tissue. *Arcobacter* and *Pseudoalteromonas* were found to be some of predominant spoilage or decomposing bacteria in oyster gills (Chen *et al.* 2019), but this does not rule out their potential to be opportunistic

oyster pathogens. In conclusion, despite differences in OsHV-1 microvariant and temperature thresholds, similar bacteria are found to associate with OsHV-1 induced disease across many studies. *Arcobacter*, *Vibrio* and *Amphritea* and *Pseudoalteromonas* are most likely to be interconnected with OsHV-1 induced disease and further research should follow up on their potential to work in concert with OsHV-1 to kill oysters.

Conclusion:

In the present study, mortality of oysters was significantly impacted by the interaction between temperature and OsHV-1 exposure. The San Diego Bay OsHV-1 microvariant can infect, but not kill, oysters when temperatures are 15°C or below. OsHV-1 can kill oysters faster at 21 and 24°C, which are typical summer temperatures experienced in San Diego Bay. Bacterial communities are significantly altered by OsHV-1 exposure but suppression of bacteria did not significantly lower mortality rates. OsHV-1 may shape bacterial community structure by altering host immune response, leading to an initial increase in bacterial richness followed by a dominance of a few bacteria. The microbiome composition is predictive of disease status, likely more so than visual observation of slow valve closing symptoms. Similar bacteria (*Arcobacter*, *Vibrio* and *Amphritea* and *Pseudoalteromonas*) were found to associate with OsHV-1 induced disease across this and multiple other studies despite differences in OsHV-1 microvariants and temperature thresholds. Overall, this study determined that temperature is important for predicting OsHV-1 San Diego Bay microvariant induced mortality, but it is unknown which factors instigate a transmission to previously unexposed oysters in natural conditions in San Diego Bay. This study also importantly recognized a high abundance of 4 conserved taxa which are almost always detected in OsHV-1 exposed oysters but had not previously been demonstrated for the San Diego microvariant and oysters from California. Future research should further investigate the interactions between these bacterial taxa and OsHV-1 to better understand their roles in oyster mortality and disease progression.

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Conflict of Interest:

We have no conflicts of interest to disclose.

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Data Availability: All 16S rRNA gene amplicon DNA sequences are deposited publicly in the European Nucleotide Archive at study accession [PRJEB72643](https://www.ebi.ac.uk/ena/browser/view/PRJEB72643). The scripts for analyzing data generated in this study are publicly available at <https://github.com/ekunselman/OsHV-1>.

Author Contribution Statement:

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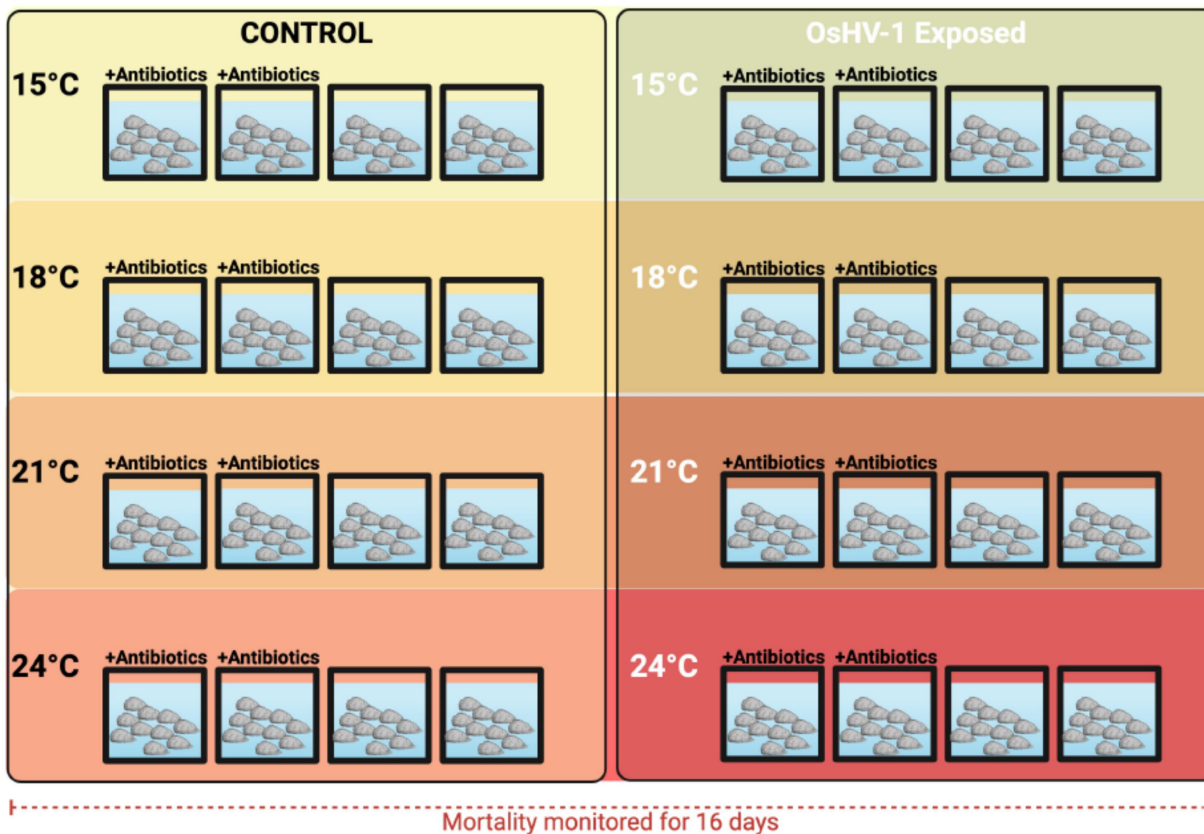
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Oyster Infection Experiment #1 - Mortality:



Oyster Infection Experiment #2 - Microbiome:

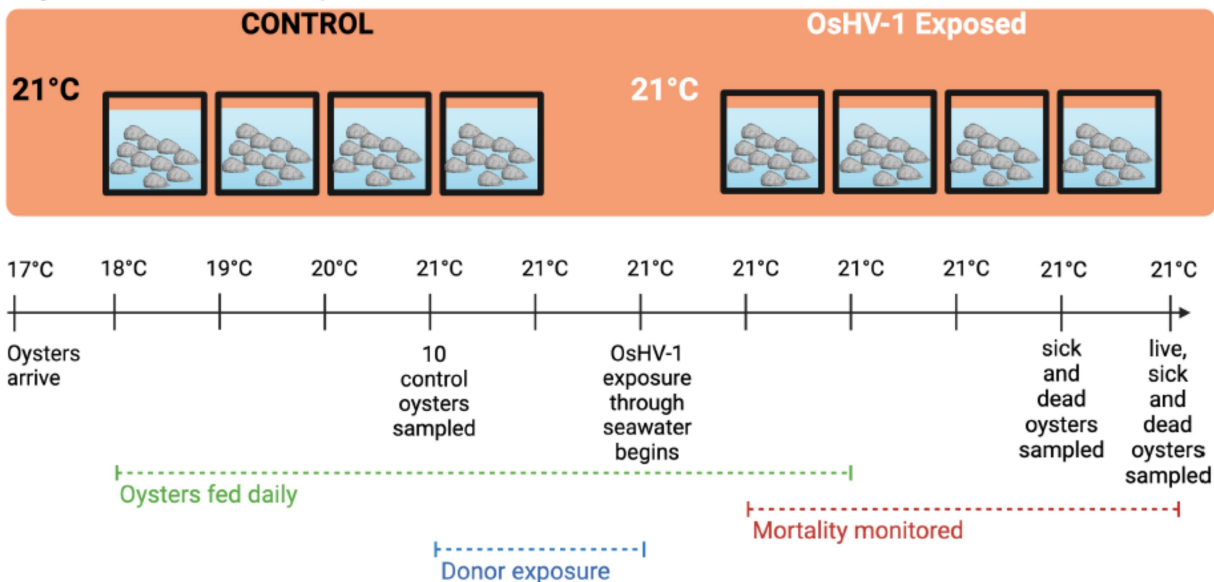
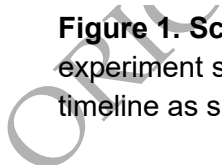


Figure 1. Schematic diagram of the experimental design. Top panel is showcasing mortality experiment setup while bottom panel is showing microbiome experiment setup. A highly similar timeline as shown in the bottom panel was used for both experiments, with the exception that



mortality was monitored for 16 days in the first experiment and oysters were not sampled until they died, while they were all sampled after 4 days in the second experiment.

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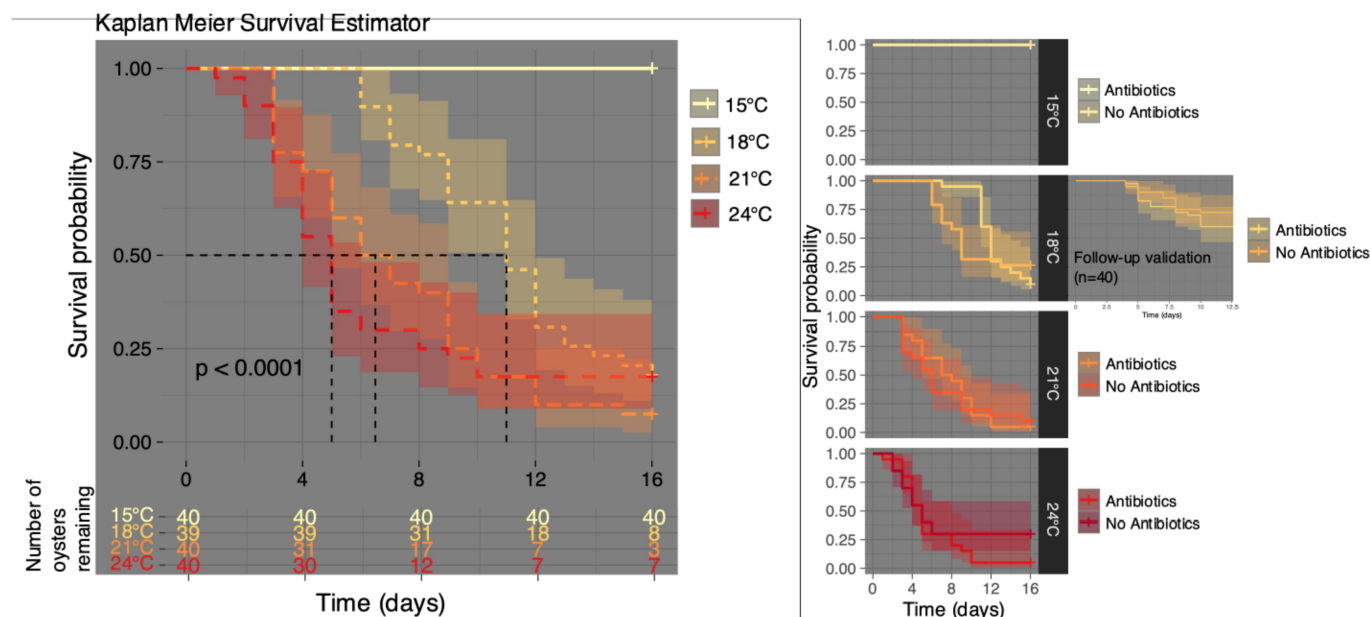


Figure 2. Kaplan Meier mortality curves displaying the proportion of oysters dead in each condition each day and estimated probability of survival with 95% confidence intervals.

Top panel displays survival curves by temperature with an x axis of time in days. Significance of the statistical difference between temperatures is exhibited with p value < 0.0001 . The number of oysters remaining at given time points is displayed below the Kaplan Meier plot, where the initial sample size of oysters is the first number listed (n = 40 for 15, 21 and 24°C, n = 39 for 18°C). Bottom panel displays survival curves by antibiotic exposure, faceted into one plot per temperature. For 18°C, the follow-up validation experiment with 40 oysters is coupled with the original experiment to show trends from the duplicated experiments.

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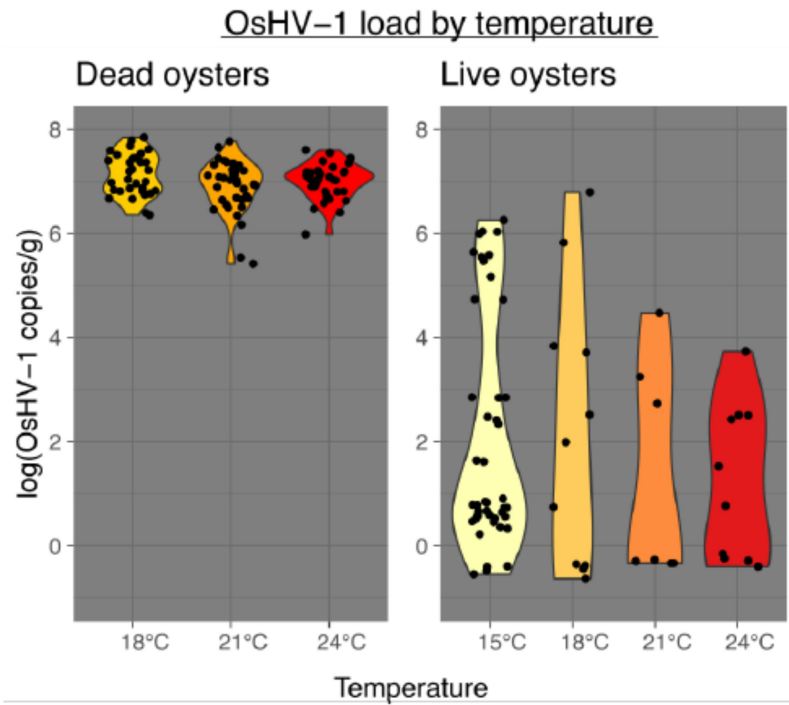


Figure 3. Viral load of OsHV-1 copies normalized by weight across temperatures faceted by mortality. Copy numbers were determined by qPCR and each individual dot represents one sample. Violin plots are used to summarize density distribution of values across temperatures. Left panel displays normalized qPCR values for dead oysters sampled throughout the experiment, while the right panel displays values for remaining live oysters collected only at the end of the experiment. 15°C is not included in the left panel as no oysters died at this temperature.

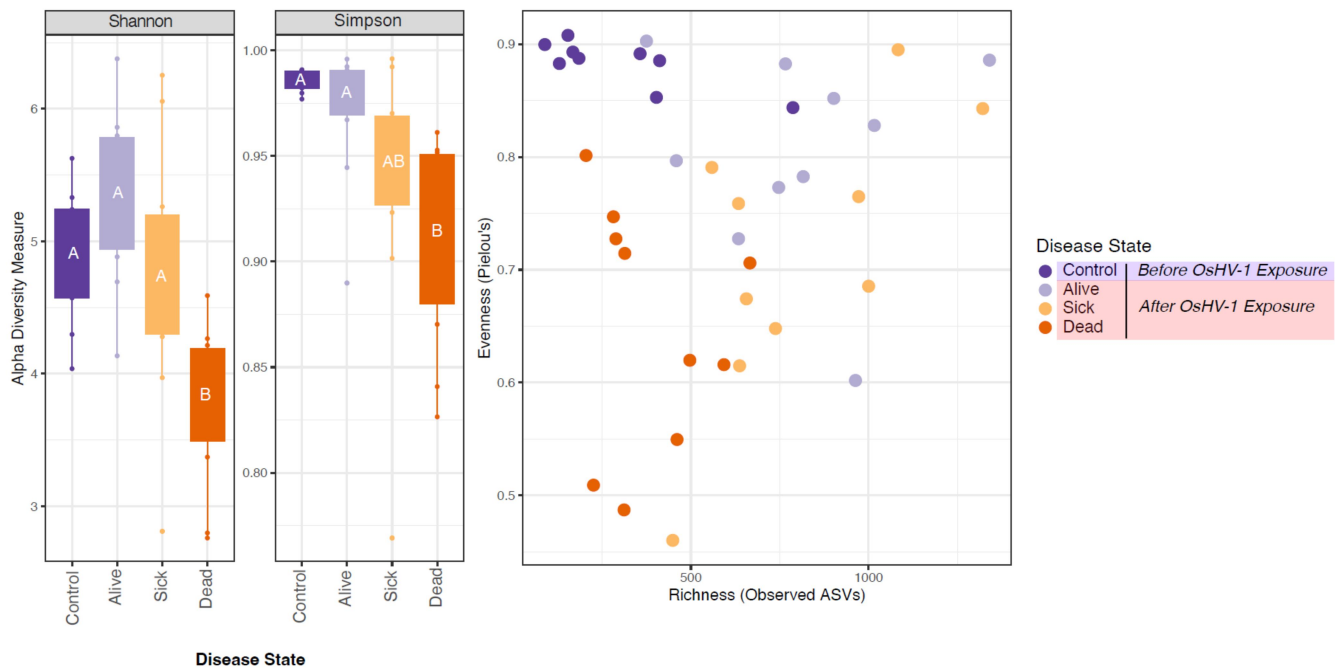


Figure 4. Alpha Diversity of oysters before and after exposure to OsHV-1. Left displays Shannon and Simpson diversity with boxplots corresponding to 4 different disease states: 1: before OsHV-1 exposure, 2: after exposure but still alive, 3: after exposure and showing signs of sickness and 4: completely dead. Statistically significant difference ($p < 0.05$) between groups is denoted by “A”, “AB”, and “B”, with “AB” suggesting overlap with both “A” and “B”. Right displays the richness in number of observed ASVs versus the evenness as measured by Pielou’s index as a dotplot. Dots are colored by their respective disease states.

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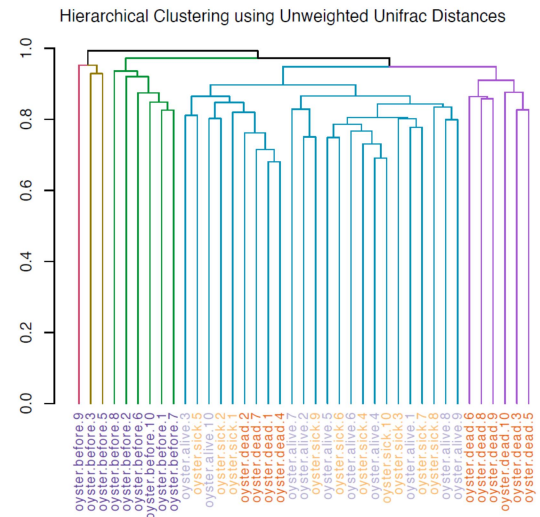
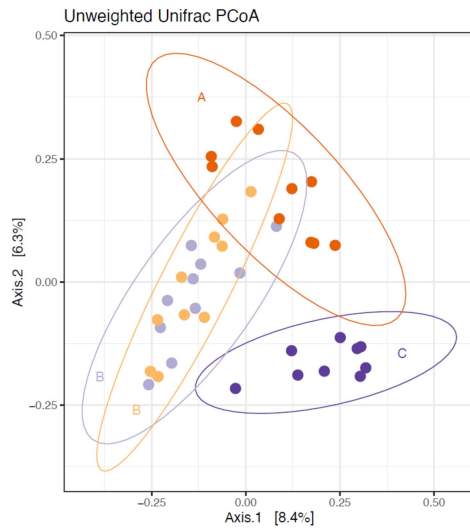


Figure 5. Beta Diversity Distances between Disease States. Left displays a Principal Coordinate Analysis of the Unweighted UniFrac distances between samples. Dots are each a single sample color-coded by Disease State and ellipses are added around the groups to demonstrate overlap. Significant differences are denoted by groups “A”, “B”, and “C”. Right displays hierarchical clustering of samples based on the Unweighted UniFrac dissimilarity matrix using the complete linkage method. Sample names are listed along the bottom and color-coded by Disease State. The colors of the lines indicate the 5 most prevalent clustering groups.

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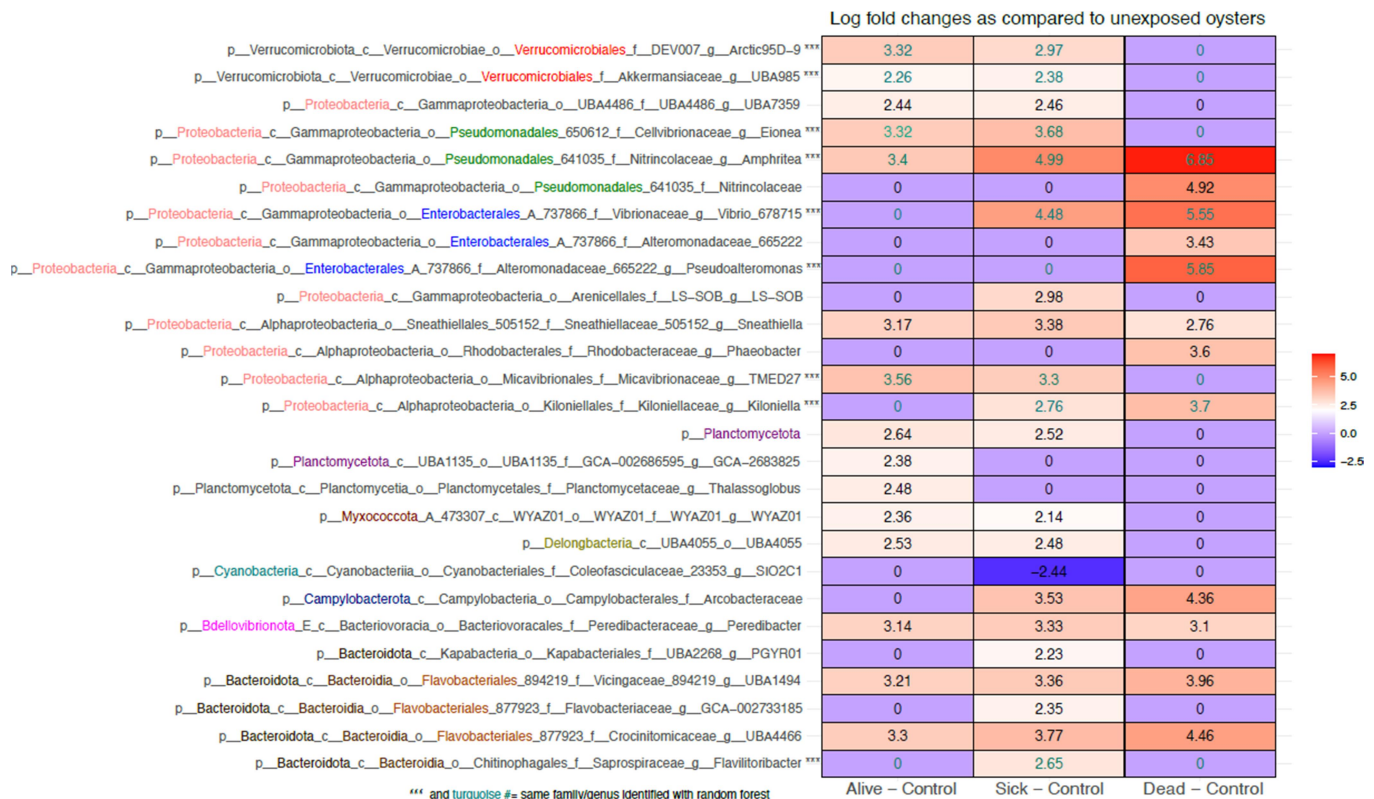


Figure 6. Differential abundance analysis of taxa changing significantly from before to after OsHV-1 exposure. Taxonomic classification down to genus level is provided on the left side of the plot. Names are colored by their taxonomic group to show similar phyla, classes, and/or orders. The log fold change of the taxa from A: Control to Alive, B: Control to Sick, and C: Control to Dead is listed in order from left to right. The value of log fold change is written in its respective box for each comparison and taxa, and the box is colored along a gradient scale from negative to positive values, with a center around 2. Numbers in the blue-green color and taxa names ending with 3 asterisks *** indicate that this genus was also identified as one of the top 20 most important in Random Forest analysis for predicting disease state, which are listed in Table 1.

Table 1. Top 20 most important ASVs for classifying oyster samples in Random Forest analysis. ASVs are listed in order of increasing importance. Genus and Species are italicized. Red lettering denotes the same ASV in both “Before versus After” and “Alive versus Dead” lists. The three asterisks *** indicates that the Genus was identified in both Random Forest and ANCOM-BC analysis.

TOP 20 ASV PREDICTORS FOR BEFORE VERSUS AFTER OSHV-1 INFECTION	
PREDICTOR CLASSIFICATION	Mean Decrease Gini
Bacteroidota Bacteroidia Flavobacteriales_877923 Flavobacteriaceae <i>Tenacibaculum_A_805184</i>	0.1641
Verrucomicrobiota Verrucomicrobiae Verrucomicrobiales Akkermansiaceae <i>Uba985</i> ***	0.1734
Bacteroidota Bacteroidia Chitinophagales Saprospiraceae <i>Lewinella_A</i>	0.1774
Planctomycetota B15-G4 B15-G4 B15-G4 B15-G4 <i>Sp003644265</i>	0.1788
Bacteroidota Bacteroidia Flavobacteriales_877923 Flavobacteriaceae <i>Patiriiisocius_Sp000170815</i>	0.1891
Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae <i>Roseovarius_489432</i>	0.2040
Campylobacterota Campylobacteria Campylobacterales Arcobacteraceae <i>Arcobacter_474983</i>	0.2098
Bacteroidota Bacteroidia Chitinophagales Saprospiraceae <i>Flavilitoribacter</i> *** <i>Nigricans</i>	0.2135
Planctomycetota B15-G4 B15-G4 B15-G4 B15-G4 <i>Sp003644265</i>	0.2186
Planctomycetota Planctomycetia Pirellulales Pirellulaceae <i>Mariniblastus_Sp011087765</i>	0.2268
Bacteroidota Bacteroidia Flavobacteriales_877923 Flavobacteriaceae	0.2348
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Vibrionaceae <i>Vibrio_678715</i> ***	0.2404
Proteobacteria Alphaproteobacteria Rhizobiales_A_501396 Rhizobiaceae_A_499470	0.2571
Proteobacteria Alphaproteobacteria Kiloniellales Kiloniellaceae <i>Kiloniella</i> ***	0.2871
Proteobacteria Gammaproteobacteria Pseudomonadales_641035 Nitrospirales <i>Amphritea</i> *** <i>Spongicola</i>	0.2910
Verrucomicrobiota Verrucomicrobiae Verrucomicrobiales Dev007 <i>Arctic95d-9</i> ***	0.2994
Proteobacteria Alphaproteobacteria Sphingomonadales Emcibacteraceae <i>Paremcibacter_Congregatus</i>	0.3072
Proteobacteria Gammaproteobacteria Pseudomonadales_650612 Cellvibrionaceae <i>Eionea</i> ***	0.3570
Proteobacteria Alphaproteobacteria Micavibrionales Micavibrionaceae <i>Tmed27</i> *** <i>Sp002167715</i>	0.3641
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Kangiellaceae <i>Aliikangiella_737838</i> <i>Coralliicola</i>	0.4182

TOP 20 ASV PREDICTORS FOR ALIVE VERSUS DEAD OYSTERS (FOLLOWING OSHV-1 INFECTION)

PREDICTOR CLASSIFICATION	Mean Decrease Gini
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Alteromonadaceae_665222	0.0990
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Alteromonadaceae_665222 <i>Paraglaciicola</i>	0.1000
Bacteroidota Bacteroidia Chitinophagales Saprosiraceae <i>Jaautg01</i> <i>Sp012031785</i>	0.1145
Bacteroidota Bacteroidia Flavobacteriales_877923 Cryomorphaceae	0.1274
Planctomycetota Brocadia Brocadiales	0.1400
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Alteromonadaceae_665222 <i>Pseudoalteromonas</i> *** <i>Phenolica</i>	0.1417
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Shewanellaceae_666538 <i>Shewanella</i> <i>Colwelliana</i>	0.1442
Campylobacterota Campylobacteria Campylobacterales Arcobacteraceae	0.1450
Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae <i>Roseovarius_489432</i>	0.1543
Bacteroidota Bacteroidia Flavobacteriales_877923 Flavobacteriaceae	0.1583
Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae	0.1720
Calditrichota Calditrichia Rbg-13-44-9 J042 J075 <i>Sp003695285</i>	0.1742
Proteobacteria Gammaproteobacteria Enterobacterales_A_737866 Vibrionaceae <i>Vibrio_678715</i> *** <i>Hanmi</i>	0.1763
Planctomycetota Gca-002687715 Gca-002687715 Gca-002687715 Gca-2683135 <i>Sp002683135</i>	0.1772
Proteobacteria Gammaproteobacteria Xanthomonadales_616050 Marinicellaceae <i>Marinicella</i> <i>Sediminis</i>	0.1829
Proteobacteria Gammaproteobacteria Pseudomonadales_641035 Nitrospiraceae <i>Amphritea</i> *** <i>Spongicola</i>	0.1980
Verrucomicrobiota Verrucomicrobiae Verrucomicrobiales Akkermansiaceae <i>Uba985</i> ***	0.1990
Proteobacteria Gammaproteobacteria Pseudomonadales_650612 Pseudohongiellaceae <i>Uba9145</i>	0.2765
Bacteroidota Bacteroidia Flavobacteriales_877923 Flavobacteriaceae <i>Maritimimonas</i> <i>Rapae</i>	0.3192
Proteobacteria Gammaproteobacteria Pseudomonadales_650612 Porticoccaceae <i>Porticoccus</i>	0.4142

ORIGINAL UNEDITED MANUSCRIPT