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Impact of seawater temperature on the Pacific oyster (*Crassostrea gigas*) microbiome and susceptibility to disease associated with *Ostreid herpesvirus-1* (OsHV-1)

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ABSTRACT

Context. Intertidal estuarine environments expose oysters to wide temperature variations. This can shift their microbiome composition towards pathogen-dominated communities. Understanding the impact of temperature on the microbiome will facilitate oyster health management. **Aims.** The present study was conducted to (1) assess the Pacific oyster microbiome at different temperatures (21°C, 22°C, 26°C and diurnal fluctuation between 22°C and 26°C) and (2) investigate microbiome changes in response to exposure to the pathogen *Ostreid herpesvirus-1* (OsHV-1) at different temperatures. **Methods.** Pacific oysters (15 months of age; $n = 480$) were acclimated to different temperatures in laboratory aquaria. Samples were obtained before and after acclimation and after OsHV-1 exposure for quantification of OsHV-1, total bacteria and *Vibrio*, by quantitative PCR. Bacterial *16S* rRNA gene (V1–V3) sequences were used to characterise the gill bacterial community. **Key results.** The alpha diversity (number of observed amplicon sequence variants) and total number of bacteria associated with the gills of oysters did not change with acclimation to different water temperature profiles, but there was variation in beta diversity. The highest mortality after OsHV-1 exposure occurred at 26°C and these, together with oysters at 22/26°C, had a higher concentration of OsHV-1 DNA compared with to the ones at the lower constant temperatures ($P < 0.05$). The total bacterial quantity increased after the OsHV-1 challenge in oysters at 22/26°C. The alpha diversity of microbiota increased after the OsHV-1 challenge in oysters at 21°C and 22/26°C. The beta diversity changed both after acclimation and OsHV-1 challenge. The highest abundance of *Vibrio* and higher OsHV-1 loads were seen in OsHV-1-challenged oysters at 26°C ($P < 0.05$). **Conclusions.** The gill microbiome altered with seawater temperature and OsHV-1 challenge. Higher mortality following OsHV-1 exposure was associated with a higher water temperature and greater abundance of *Vibrio* spp. arising from the microbiome. **Implications.** Higher seawater temperature can be considered a key risk factor influencing oyster health by altering the microbiome, increasing susceptibility to OsHV-1 and increasing the *Vibrio* fraction in the oyster microbiome.

Keywords: *Crassostrea gigas*, estuarine, intertidal, microbiome, OsHV-1, *Ostreid herpesvirus-1*, Pacific oyster, temperature.

Introduction

Host-associated microbiomes are generally diverse, dynamic and respond to various internal factors such as the age of host, within-microbiome interactions as well as external factors (Ley *et al.* 2008; Yatsunenko *et al.* 2012; Shade *et al.* 2013; Mancuso *et al.* 2016; Meyer *et al.* 2016; Adair and Douglas 2017). The Pacific oyster microbiome is influenced by the environment as these animals are ectothermic and do not possess an adaptive immune system (Green *et al.* 2014; Lokmer *et al.* 2016*b*). The Pacific oyster microbiome changes in response to changes in pH (Flores-Higuera *et al.* 2019), seawater temperature (Lokmer and Wegner 2015), temperature stress (Lokmer and Wegner 2015) and locality (Pathirana *et al.* 2019*b*).

Temperature is a key factor that influences bacterial growth and persistence (Zwietering *et al.* 1994). Water temperature is a strong determinant of bacterial populations inhabiting seawater (Lokmer *et al.* 2016; Lokmer and Wegner 2015) and an important external factor influencing host-associated microbiomes (Lokmer and Wegner 2015; Roterman *et al.* 2015). Disruption of core microbial communities can lead to an increase in rare microbial taxa, resulting in increased heterogeneity in the microbiome composition with increased seawater temperatures (Erwin *et al.* 2012). The core microbiome comprise microbial taxa that are in high prevalence in a host population or species (Adair and Douglas 2017). In marine sponges, the symbiotic microbial community can be replaced by pathogenic microbes in the event of elevated seawater temperature (Maldonado *et al.* 2010; Cebrian *et al.* 2011). Higher heterogeneity of the microbiome was observed in spondylus oysters (*Spondylus spinosus*) in summer when the seawater temperature was higher than 30°C (Roterman *et al.* 2015). In contrast, several studies have observed loss of bacterial diversity in the oyster microbiome in response to diverse stress factors (Lokmer and Wegner 2015; Lokmer *et al.* 2016*a*; Lasa *et al.* 2019). In addition to the microbiome dynamics, temperature increases have been linked to enhanced disease expression by increasing pathogen development and host susceptibility to disease (Harvell *et al.* 2002; Burge *et al.* 2014). Disease outbreaks in Pacific oysters caused by *Ostreid herpesvirus-1* (OsHV-1) have been associated with elevated seawater temperatures (Garcia *et al.* 2011; Paul-Pont *et al.* 2014; Whittington *et al.* 2019).

Pacific oyster mortality events associated with OsHV-1 infection occur when environmental conditions favour development of disease (Petton *et al.* 2015; de Kantzow *et al.* 2017; Evans *et al.* 2019). The severity of disease is influenced by environmental factors such as elevated seawater temperature, salinity, pH and nutrient concentrations in seawater (Soletchnik *et al.* 2007; Petton *et al.* 2013; de Kantzow *et al.* 2016; Delisle *et al.* 2018). In Europe, OsHV-1 disease outbreaks have usually occurred at seawater temperature at or above 16°C (Pernet *et al.* 2012; Clegg *et al.* 2014; Renault *et al.* 2014; Petton *et al.* 2015) and oyster mortality does not occur at temperatures above 26°C (Pernet *et al.* 2012). However, this disease in Australia occurred with water temperatures 4–5°C warmer than those recorded in Europe (Paul-Pont *et al.* 2014; Whittington *et al.* 2019). Importantly, surveillance indicates that OsHV-1 infection does not always lead to mortality at the conducive temperatures (Whittington *et al.* 2019).

During summer, oysters living in intertidal estuarine environments are exposed to diurnal temperature fluctuations, including high temperatures when out of the water column. Heat stress can favour shifts in the microbiome towards pathogen-dominated communities (Boutin *et al.* 2013). Increased seawater temperatures (>20°C) have also been associated with Pacific oyster mortality outbreaks in Port Stephens, New South Wales (NSW), in the absence of OsHV-1 (Go *et al.* 2017). Microbiome analysis of affected oysters from this outbreak showed an increase of rare microbiota (low-abundant bacteria in the microbiome) belonging to the genus *Vibrio* (King *et al.* 2019*a*). Green *et al.* (2019) demonstrated that mortality triggered by increasing seawater temperature to 25°C was reduced (from 77.4% to 4.3%) when the oysters were treated with the broad-spectrum antibiotic combination of penicillin and streptomycin, indicating a role of bacteria in temperature-associated mortality. While the increase of temperature was associated with a 324-fold increase of *Vibrio harveyi*, a reduction of *Vibrio harveyi* from 40.5% to 2.2% was reported after the antibiotic treatment. There is an emerging view of polymicrobial pathogenesis for Pacific oyster mortality triggered by an immunosuppressive effect of OsHV-1 infection (de Lorgeril *et al.* 2018), or, indeed, microbiome disruption due to other stressors (King *et al.* 2019*b*; Lasa *et al.* 2019).

Understanding the impact of seawater temperature on the oyster microbiome may lead to new ways of controlling oyster mortality. The aims of the current study were to assess the Pacific oyster microbiome at different temperatures (21°C, 22°C, 26°C and diurnal fluctuation between 22°C and 26°C), and, further, to investigate microbiome changes in response to *Ostreid herpesvirus-1* (OsHV-1) exposure at different temperatures in a controlled laboratory environment and determine a potential role of the microbiome in the disease outcome.

Materials and methods

Oysters

Hatchery-reared, single-seed, triploid Pacific oysters were used in this study (Batch SPL17C; Shellfish Culture Ltd, Tasmania; n = 480). They were grown under commercial farming conditions in the Hawkesbury River, NSW (Broken Bay Oysters), to 15 months of age (shell length 50–80 mm; weight 17.5–44.5 g). The oysters were free from OsHV-1 on the basis of negative quantitative PCR (qPCR) tests for a random sample (n = 30) when transported to a physical containment level 2 (PC2) aquatic animal facility at the University of Sydney.

The Pacific oyster is not considered by the *NSW Animal Research Act 1985* nor the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes, 8th edition (2013). Therefore, approval from the Animal Ethics Committee, University of Sydney, was not required for this study.

Experimental design and aquarium management

After bringing into the PC2 facility, the oysters were purged overnight in artificial seawater (ASW; Red Sea® salt) and

randomly allocated to 24 individual tanks (Fig. 1) containing 12 L of aerated ASW at 30 ± 1 ppt (g of salts/kg of seawater) salinity ($n = 20$ oysters/tank). The water temperature in tanks was maintained using external water baths contained in four separate recirculation systems, each dedicated for one temperature treatment. The temperature of the water bath was maintained by the combined actions of thermostatically controlled aquarium heaters (AquaOne®, NSW, Australia); aquarium heater chiller units (HC-300a, Hailea Aquarium chiller); and air temperature maintained at 24°C ± 2°C by air conditioning.

Oysters were acclimated to the tank environment for a total of 14 days, including a period of temperature adjustment. Six tanks were allocated to each temperature profile: constant at 21°C, 22°C, or 26°C and diurnal variation between 22°C and 26°C. This dynamic temperature profile was achieved by setting the heater and chiller units to 26°C at 9 a.m. each morning and 22°C at 6 p.m. each evening. During acclimation, the water temperature in all tanks was set at 20°C for 2 days. For the static 21°C, 22°C and 26°C treatments, the temperature was increased by 1°C/day until the target temperature was reached. For the 22/26°C dynamic temperature, the highest temperature in the cycle was increased by 1°C/day, until it reached 26°C. Temperature data loggers (Thermocron) were placed in two randomly selected tanks of each temperature to record the water temperature every 30 min. The water temperature profiles in the experimental design were achieved as follows: mean water temperature, constant 21°C, 20.9 ± 0.07°C; constant 22°C, 22.5 ± 0.02°C; constant 26°C, 25.9 ± 0.16°C; diurnal variation between 22°C and 26°C; lower temperature, 21.7 ± 0.08°C, higher temperature 25.1 ± 0.23°C.

Oysters were fed a maintenance ration (2 mL/tank.day) of commercial algae concentrate (Shellfish Diet 1800, Reed Mariculture). Total ammonia nitrogen (TAN) and pH in tank water were measured in opportunistically selected tanks of each temperature treatment, every day (API® Marine Saltwater Master Test kit). The water quality was maintained at target levels (TAN < 2 ppm, pH range 8.0–8.2) by using water exchange.

Challenge with OsHV-1

The oysters were challenged with OsHV-1 by injection of 1.34×10^6 OsHV-1 genome copies in 100 µL per oyster. The preparation was a cryopreserved, 0.2 µm filtered oyster tissue homogenate that contained OsHV-1 from diseased oysters diluted in sterile ASW as previously described (Evans *et al.* 2015). Oysters were first immersed in a solution of $MgCl_2$ (50 g/L) for 4–6 h until relaxation to facilitate the

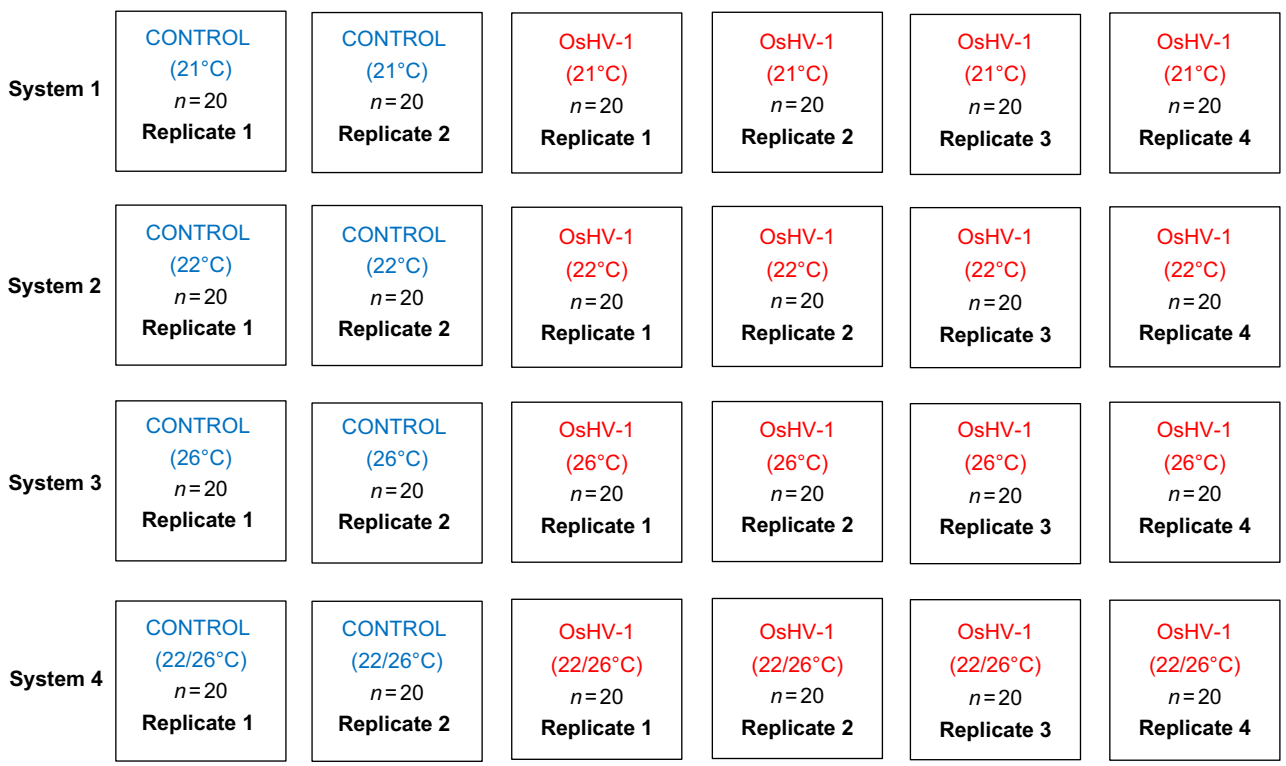

Fig. 1. Schematic representation of the experiment design, showing the allocation of oysters (*C. gigas*) across the four temperature profiles (constant at 21°C, 22°C, 26°C and diurnal fluctuation between 22°C and 26°C). Each square indicates an individual replicate tank that housed 20 oysters at the beginning of the experiment. Each system consisted of two control tanks in which the oysters were injected with an OsHV-1-negative tissue homogenate when the oysters in the other tanks were injected with OsHV-1.

injection into the adductor muscle. Two tanks from each temperature treatment were allocated for injection with the negative control oysters. The negative control was a tissue homogenate prepared and injected in the same way from apparently healthy, OsHV-1-free Pacific oysters (Evans *et al.* 2015). The water temperature was 26°C in the tanks with the dynamic temperature profile at the time of OsHV-1 injection.

Sampling

Oysters were randomly sampled before ($n = 12$) and after the 14-day acclimation ($n = 48$; two oysters from each tank). Sampling was repeated 48 h after OsHV-1 challenge ($n = 48$; two oysters per tank per temperature profile). Oysters were inspected twice daily, and any dead oysters were sampled immediately. All oysters that survived the infection challenge and the remaining negative control oysters were sampled on Day 10 post-injection. Oysters were held at 4°C until tissue dissection within 1 h of sampling. The cumulative mortality for each group was calculated according to the method described by Whittington *et al.* (2015), which accounted for the number of live oysters sampled during the trial.

Molecular quantification of OsHV-1

A previously described method was used to detect and quantify OsHV-1 in all sampled oysters (Pathirana *et al.* 2019*b*). Briefly, equal portions of gill and mantle tissue (0.08–0.12 g in total) were excised from each oyster, homogenised by bead-beating and nucleic acids were extracted using the Ambion MagMax™-96 Viral RNA Isolation Kit with a BioSprint-96™ magnetic particle processor (Qiagen).

The number of copies of the B-region of the OsHV-1 genome was determined relative to a plasmid DNA standard according to Paul-Pont *et al.* (2013) using a qPCR assay (Martenot *et al.* 2010). Samples were tested in duplicate reactions prepared with Path-ID qPCR master mix (Life Technologies) and tested using a Mx3000P Multiplex Quantitative PCR System (Stratagene).

Nucleic acid extraction for molecular bacterial studies

Approximately 30 mg from the gill of each oyster was collected separately for nucleic acid purification using the E.Z.N.A.® Mollusc DNA kit (Omega Bio-Tek, USA) according to the approach detailed by Pathirana *et al.* (2019*a*). The qPCR assay described by Vezzulli *et al.* (2012) for quantification of *Vibrio* spp. DNA was used as described by Pathirana *et al.* (2019*b*). Similarly, the qPCR assay described by Nadkarni *et al.* (2002) was adapted to quantify total bacteria in gill tissues as described in Pathirana *et al.* (2019*b*).

Microbiome analysis by high-throughput *16S* rRNA gene sequencing

Nucleic acid extracts from gill tissues ($n = 94$) were selected to represent oysters before acclimation, from all four temperature treatments, and OsHV-1-challenged and negative control oysters from each temperature treatment. The microbial community composition of each extract was identified by high-throughput sequencing of the hypervariable region V1–V3 of the *16S* rRNA gene. DNA sequencing was performed by the Ramaciotti Centre for Genomics, University of New South Wales, Australia. PCR amplicons were generated using primers 27F and 519R (Lane 1991). The amplification was performed in reaction mixtures containing 200 μM of each dNTP, 0.5 μM of each primer, 1 μL of template DNA, one unit of Immolase DNA Polymerase, 2.5 μL of 10 × Immolase Buffer (contains 2.5 mM $MgCl_2$) and sterile, nuclease-free water. The PCR program was as follows: initial denaturation of 95°C for 10 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 10 s and elongation at 72°C for 45 s; final elongation of 72°C for 10 min. Cleaned PCR products were subjected to quality control by using Qubit and Tapestation, followed by sequencing on the Illumina MiSeq System with 300-bp paired-end chemistry. The sequencing run also carried a positive control and a negative control. The quality of the raw sequence data was assessed with FASTQC (Andrews 2010). A metadata file for the samples was generated in Microsoft Excel and was validated using the browser-based metadata validation tool, Keemei (Rideout *et al.* 2016). The software suite, Quantitative Insights into Microbial Ecology 2 (QIIME2; 2018.11 release), was used in the analysis of these sequence data. Demultiplexed, paired-end DNA sequence reads were imported into the QIIME2 pipeline and the Divisive Amplicon Denoising Algorithm 2 (DADA2) was used to reduce noise, remove replication and chimera-filter the reads (Callahan *et al.* 2016). Using the DADA2 pipeline, the sequence reads were truncated at 120 bp on the basis of -the quality of sequence data and the reverse reads were reverse-complemented and concatenated with the forward reads. There is a possibility of reducing microbiome diversity estimates through exclusion of sequence reads that fail to merge (Aigle *et al.* 2019; Dacey and Chain 2021). Concatenating paired reads together without a sequence overlap has been identified as an alternative to overcome this problem (Aigle *et al.* 2019; Dacey and Chain 2021).

A feature table was created with the number of reads for each unique sequence and mapped feature identifiers to the sequences they represent. The taxonomic analysis was undertaken using a naïve-Bayesian classifier (QIIME2 sklearn) trained using Greengenes (v.13.8) 99% ASVs, where the sequences were trimmed to include only the V1–V3 hypervariable region of *16S* rRNA gene (McDonald *et al.* 2012). The relative abundance of bacterial phyla in each sample was graphically represented using 100%

stacked 2-D column graphs (Excel, Microsoft). The microbial diversity (alpha and beta diversity) analyses were performed using the q2-diversity plugin of QIIME2 (Bokulich *et al.* 2018). The number of observed ASVs was used as the parameter to assess alpha diversity of samples. The dissimilarity of bacterial community structure among samples (beta diversity) was visualised by principal coordinate plots based on the two-dimensional Bray–Curtis dissimilarity index.

Statistical analyses

Kaplan–Meier survival curves and Cox regression analyses were used to investigate the differences in survival among oysters with different temperature profiles after the OsHV-1 challenge (SPSS Statistics ver. 22; IBM SPSS Corp., Somers, NY, USA). The quantity of OsHV-1 DNA and total bacterial DNA were compared among groups after $\log_{10}$ transformation and assessing for normal distribution (SPSS Statistics). Univariate general linear models were used for separate analysis of OsHV-1 DNA and total bacteria DNA (GLM, SPSS Statistics). Fixed factors considered for OsHV-1 DNA were temperature profile, days after OsHV-1 challenge (Days 2–10), outcome of infection (live or dead), together with possible interactions with tank identification included as a random factor. For total bacteria, time of sampling (before and after acclimating to a temperature profile and after OsHV-1 challenge) and temperature profile were tested as fixed factors together with possible interactions, with tank included as a random factor. For both models, *posthoc* pairwise mean comparisons were made using the least significant difference method. The results are presented as geometric means and their corresponding 95% confidence intervals for OsHV-1 genome equivalents and bacterial genome equivalents per milligram respectively. As the total *Vibrio* count was below the limit of quantification for some treatment groups, it was not statistically analysed.

Alpha diversity (number of observed ASVs) of bacterial communities was analysed using the Kruskal–Wallis test and beta diversity was assessed using one-way permutational multivariate ANOVA (PERMANOVA). Variation in the absolute abundance of selected phyla and genera among treatment groups were evaluated using generalised linear models (GzLM; SPSS). Significance was set at $P < 0.05$ for all statistical analyses.

Results

Water temperature

The diurnal dynamic temperature reached a peak 8 h 56 min ± 16 min after the morning change in temperature settings, while the temperature reduced to the lower margin 3 h 46 min ± 15 min after the evening temperature adjustment.

Mortality

Mortality began on Day 2 post-injection in the OsHV-1-challenged oysters in all treatment groups, except at 21°C constant water temperature, where mortality commenced on Day 3 post-injection. While the mortality ceased after Day 7 post-injection at 26°C, it continued until Day 9 at 22°C and until Day 10 at both constant 21°C and in the 22/26°C dynamic temperature profiles. The total cumulative mortality aggregated across replicate tanks was 48.4% (21°C), 68.8% (22°C), 84.4% (26°C) and 78.1% (22°C/26°C). No mortality was recorded in any of the negative control groups. Survival analysis indicated a significantly higher survival in OsHV-1-challenged oysters at a constant temperature of 21°C than in the oysters at other temperatures (Fig. 2; $P < 0.05$). The oysters at 22°C, 26°C and 22/26°C had a hazard of death 1.70, 3.11 and 2.43 times higher than did those at 21°C respectively (Cox hazard ratios). Interestingly, the mortality of oysters at a constant temperature of 26°C was not significantly different from that at 22°C or in the 22/26°C dynamic treatment.

OsHV-1 detection and quantity

OsHV-1 was not detected in any of the oysters tested before acclimation to the laboratory environment, after acclimating to different temperature treatments or in oysters injected

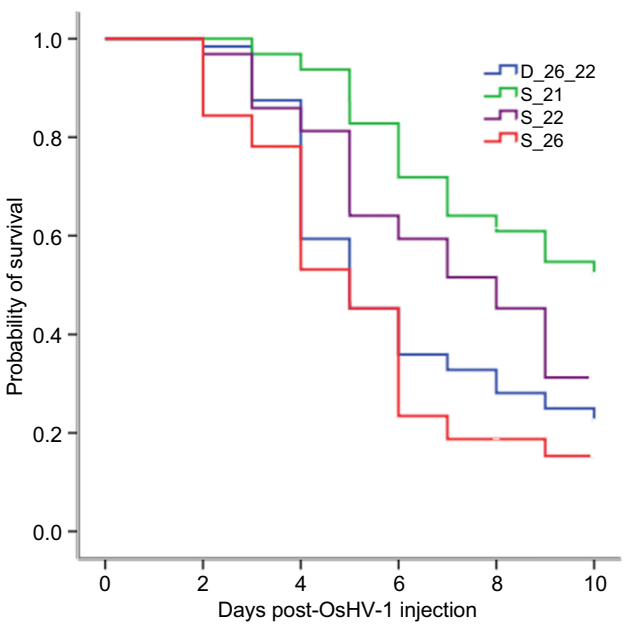

Fig. 2. Kaplan–Meier survival curves for Pacific oysters challenged with OsHV-1 and maintained with water temperature profiles of constant 21°C, 22°C or 26°C and with a diurnal fluctuation between 22°C and 26°C.

Table 1. OsHV-1 DNA concentration in gill and mantle tissues of oysters challenged with OsHV-1 and maintained at different water temperature profiles.

Parameter	Mean OsHV-1 concentration (genome equivalents/mg)		Number of oysters
	Geometric mean[A]	95% confidence interval (lower–upper)	
Temperature profile			
21°C constant	6.21×10^3a	2.27×10^3–1.70×10^4	70
22°C constant	6.30×10^3b	2.28×10^3–1.74×10^4	70
26°C constant	7.60×10^3c	2.53×10^3–2.29×10^4	69
22/26°C dynamic	2.12×10^4c	8.06×10^3–5.55×10^4	71
Status of oysters			
Live	4.97×10^2	1.02×10^2–2.42×10^3	104
Dead	2.69×10^{5B}	8.14×10^4–3.53×10^5	176

Live oysters were sampled on Day 2 and Day 10 post-injection, while the dead oysters were sampled at the time of mortality. Predicted means and their corresponding 95% confidence intervals from a general linear model (GLM) were back-transformed to obtain mean OsHV-1 genomes per mg of tissue. Mean OsHV-1 concentrations with different lower-case letters awere significantly different (at $P \leq 0.05$).
[A]Geometric means and their corresponding 95% confidence intervals were derived by back-transforming the estimated model means of a general linear model (GLM).
[B]The OsHV-1 concentration was higher in dead oysters than in live oysters ($P < 0.05$).

with an OsHV-1-negative tissue homogenate. The concentration of OsHV-1 DNA was lowest in oysters maintained at a constant 21°C water temperature and was lower in oysters at 22°C than in oysters at 26°C or with a diurnal fluctuation between 22°C and 26°C (Table 1; $P < 0.05$).

Total bacteria and total *Vibrio* quantity

The total quantity of bacterial DNA in gills after acclimation was similar for all water temperature profiles and was not different from that before acclimation to temperature treatments (Table 2). Following the OsHV-1 challenge, the

Table 2. Quantity of total bacterial DNA associated with the gill of oysters, before and after acclimation to the laboratory at different water temperature profiles and 48 h post-OsHV-1 challenge.

Treatment group	Total bacteria concentration		Number of oysters
	Geometric mean	CI; lower–upper	
Before acclimation	3.23×10^4	1.19×10^4–8.79×10^4	10
After acclimation			
21°C constant temperature	8.51×10^4	3.41×10^4–2.12×10^5	12
22°C constant temperature	3.60×10^4	1.44×10^4–8.99×10^4	12
26°C constant temperature	3.18×10^4	1.27×10^4–7.93×10^4	12
22/26°C dynamic temperature	2.21×10^4	8.85×10^3–5.51×10^4	12
After OsHV-1 challenge			
21°C constant temperature	3.18×10^4	1.14×10^4–8.87×10^4	8
22°C constant temperature	2.34×10^4	5.19×10^3–1.06×10^5	8
26°C constant temperature	2.73×10^4	1.08×10^4–6.92×10^4	8
22/26°C dynamic temperature	2.63×10^{5A}	6.30×10^4–1.10×10^6	8
OsHV-1 negative control			
21°C constant temperature	2.93×10^4	6.88×10^3–1.25×10^5	4
22°C constant temperature	2.44×10^4	2.90×10^3–2.06×10^5	4
26°C constant temperature	3.56×10^4	7.13×10^3–1.79×10^5	3
22/26°C dynamic temperature	2.09×10^4	2.77×10^3–1.58×10^5	4

The number of bacterial genomes per gram of tissue was measured using qPCR and the $\log_{10}$-transformed data were analysed using a general linear model (GLM). The predicted means and their corresponding 95% confidence intervals were back-transformed to obtain mean bacterial genomes/mg tissue, for different groups.
[A]The total bacteria concentration increased after the OsHV-1 challenge in the 22/26°C dynamic temperature treatment group.

quantity of total bacteria did not change, except for an increase in oysters with a diurnal fluctuation of water temperature (22/26°C) (Table 2; $P < 0.05$). This increase was not observed in oysters that were injected with the OsHV-1-negative tissue homogenate.

The total *Vibrio* DNA in gill was below the limit of quantification (BLOQ) of the qPCR assay for 6/10 oysters before acclimation. After acclimation, *Vibrio* DNA associated with gills increased to quantified amounts (1.87×10^4–3.66×10^6 *Vibrio* genome equivalents per gram of tissue) in oysters maintained at 26°C. However, as the *Vibrio* counts for approximately 90% of the samples from the other treatments were BLOQ, a statistical analysis could not be undertaken (the limit of detection for this qPCR assay was two *Vibrio* gene copies/PCR reaction). An alternative measure of *Vibrio* abundance was obtained using *16S* rRNA gene sequence reads.

Bacterial community composition

Targeting the hypervariable V1–V3 region of the *16S* rRNA gene, a total of 3 779 301 paired-end raw reads was obtained initially from the 94 samples analysed, leaving 3 219 256 after quality control and bioinformatic processing. The median number of reads per gill tissue sample was 28 343 (maximum: 111 722; minimum: 2772). The reads were rarefied to 19 730 per sample. Rarefaction curves showed saturation for most of the samples, indicative of a good coverage of diversity.

The bacteria associated with the gill of oysters from all treatment groups was dominated by phylum *Proteobacteria* throughout the experiment. However, changes in the bacterial communities were observed after acclimation to the laboratory with different water temperature profiles and after OsHV-1 challenge (Fig. 3).

The alpha diversity (number of ASVs) in the gill microbiome did not change after acclimation to the tank environment, irrespective of the temperature profile (Table 3). However, the bacterial community composition (beta diversity) changed after acclimation at each temperature (Fig. 4; $P < 0.05$). The absolute abundance of phylum *Tenericutes* increased in oysters that were acclimated at 21°C ($P < 0.05$). No such changes were observed at phylum level after acclimation at 26°C. At the genus level, it was particularly the genus *Mycoplasma* (phylum *Tenericutes*) that increased in abundance at 21°C (Table 4; $P < 0.05$). Meanwhile, there was a reduction in the abundance of genus *Arcobacter* (phylum *Proteobacteria*) in all treatment groups after acclimation (Table 4; $P < 0.05$). It is important to note that the *Vibrio* fraction present in gills did not change after acclimation to different temperatures.

Alpha diversity of bacteria associated with the gills of oysters injected with OsHV-1 was higher than in the negative control counterparts for the constant 21°C and dynamic 22/26°C water temperature profiles (Table 3; $P < 0.05$). Alpha diversity after OsHV-1 challenge was the same for different temperatures, except for a reduction in oysters at a constant 26°C (Table 3; $P < 0.05$).

It is interesting to note that the phyla *Proteobacteria* and *Bacteroidetes* did not change in absolute abundance after acclimating at different temperatures (Fig. 3*a*–*d*). However, compared with the negative control oysters, both phyla increased in abundance after OsHV-1 challenge, except for phylum *Bacteroidetes* at the constant 22°C and 26°C temperatures (Fig. 3*a*–*d*; $P < 0.05$). Phylum *Tenericutes* reduced in abundance after the OsHV-1 challenge at 21°C and the 22/26°C dynamic temperature (Fig. 3*a*–*d*; $P < 0.05$). This reduction also occurred in the negative control oysters at 21°C.

After the OsHV-1 challenge, changes at the genus level were an increase in the abundance of *Polaribacter* (phylum *Bacteroidetes*) at 21°C and genus *Vibrio* (phylum *Proteobacteria*) in all treatment groups (Table 4; $P < 0.05$). These changes did not occur in negative control oysters. A decrease of *Mycoplasma* (phylum *Tenericutes*) was noted in OsHV-1-injected and control oysters when the water temperature was a constant 21°C. It is interesting to note that the abundance of *Vibrio* in the gill microbiota of OsHV-1-challenged oysters at 26°C was higher than in oysters at other temperatures (Fig. 5; $P < 0.05$).

Discussion

This study evaluated the influence of seawater temperature on the Pacific oyster microbiome, with different water temperature profiles in laboratory aquaria, and the impact of OsHV-1 infection. Significant changes in the bacterial community associated with the gills occurred after acclimation to different water temperatures. These changes were different depending on the water temperature profile within the range at which OsHV-1 causes disease outbreaks (Clegg *et al.* 2014; Paul-Pont *et al.* 2014; Renault *et al.* 2014; Whittington *et al.* 2019).

The temperature 21°C was selected to represent a threshold temperature for OsHV-1 infections in Australia (Paul-Pont *et al.* 2014; Whittington *et al.* 2019), while 22°C was chosen to investigate any potential changes in the microbiome response with a subtle increase in temperature. Comparatively higher oyster mortality in OsHV-1 infection studies and the observation of as an upper threshold temperature led to the selection of 26°C (de Kantzow *et al.* 2016; Delisle *et al.* 2018). The 22°C and 26°C dynamic treatment was selected to analyse the response of the microbiome to diurnal temperature dynamics in the field.

The phylum *Proteobacteria* dominated the gill microbiome of oysters in all treatment groups, throughout the experimental period. The dominance of phylum *Proteobacteria* has previously been demonstrated in gill microbiota (Wegner *et al.* 2013; Pathirana *et al.* 2019*a*) as well as in other tissues of oysters (Hernandez-Zarate and Olmos-Soto 2006; Fernandez-Piquer *et al.* 2012; Trabal *et al.* 2012; Lokmer *et al.* 2016*b*).

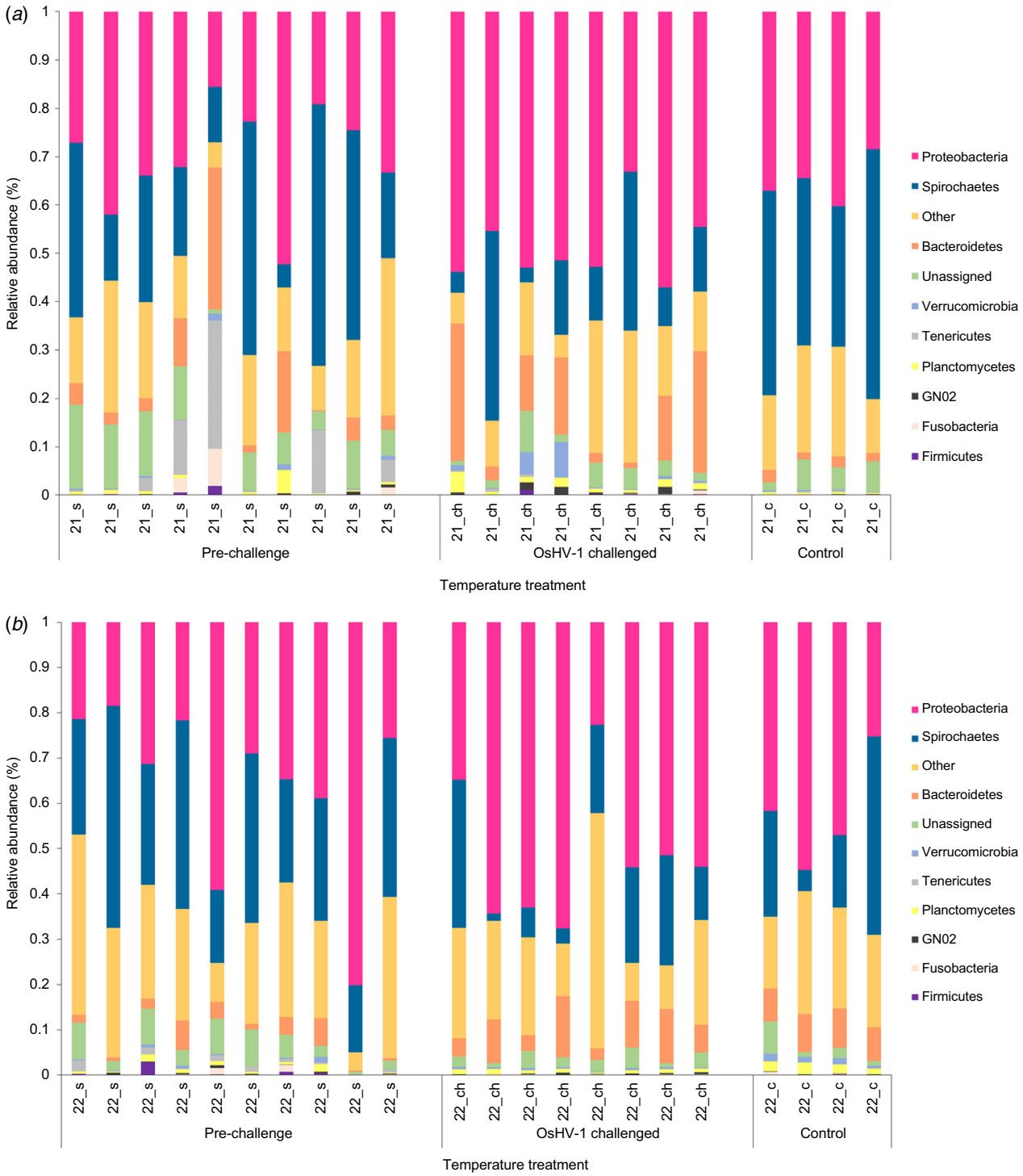

Fig. 3. Taxon bar plots indicating the relative abundance of bacterial phyla associated with the gill of oysters before and after OsHV-1 challenge and in negative control oysters maintained with the same temperature profiles. (*a*) After acclimation at constant 21°C (21_s), 21°C OsHV-1 challenged (21_ch) and negative control (21_c); (*b*) after acclimation at constant 22°C (22_s), 22°C OsHV-1 challenged (22_ch) and negative control (22_c); (*c*) after acclimation at constant 26°C (26_s), 26°C OsHV-1 challenged (26_ch) and negative control (26_c); and (*d*) after acclimation at diurnal fluctuation between 22°C and 26°C (22/26_d), 22/26°C OsHV-1 challenged (26/22_ch) and negative control (26/22_c). Live oyster samples were collected 14 days after acclimation to the relevant temperatures, and 48 h after the OsHV-1 challenge. The negative control oysters were sampled at the same time as were OsHV-1-challenged oysters. Bacteria that could not be assigned to a particular phylum are categorised under 'unassigned', and phyla with a relative abundance of less than 5% and were not present in at least two samples are categorised as 'other'.

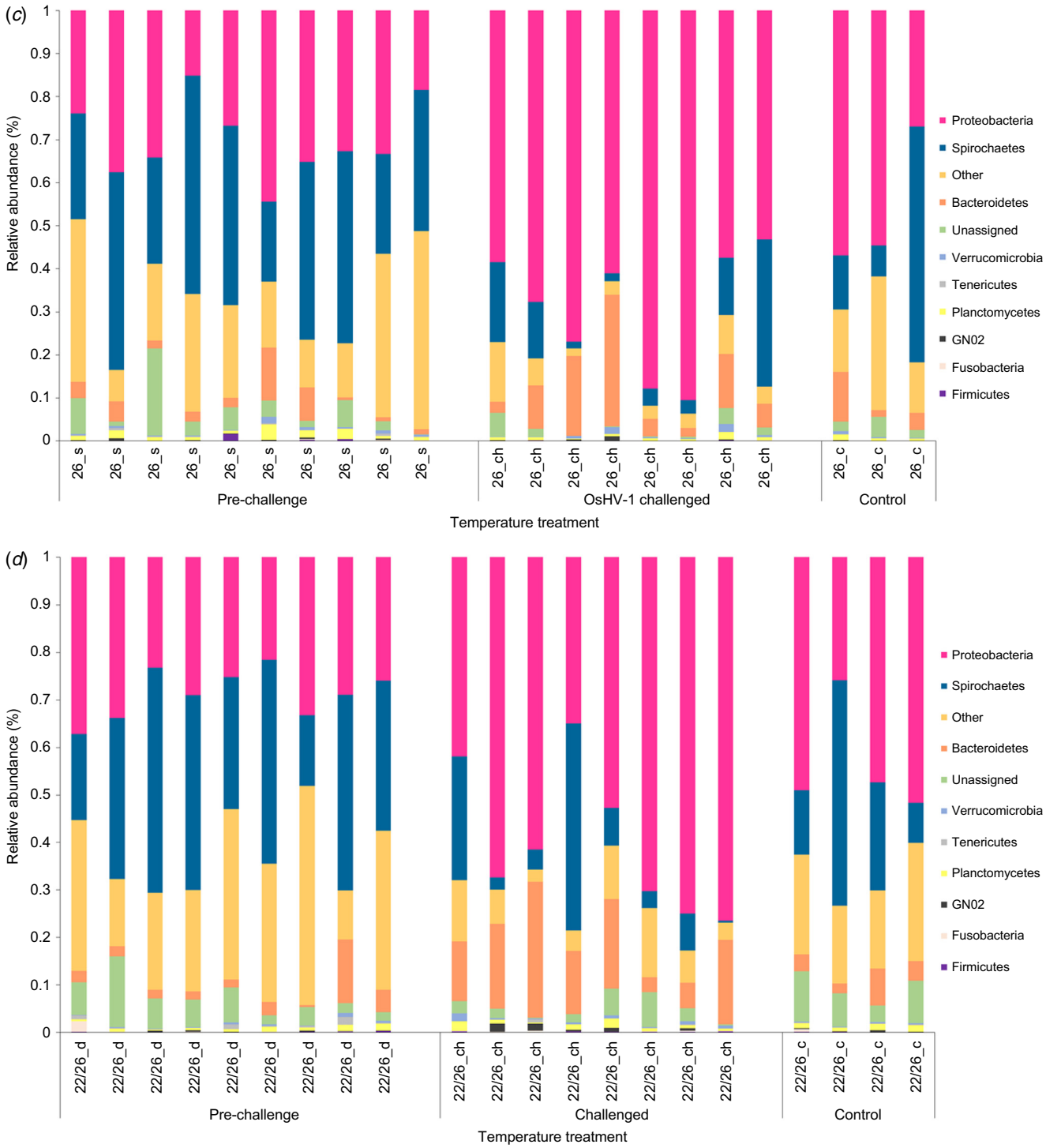

Fig. 3. (*Continued*).

The present study demonstrated that the phyla *Proteobacteria* and *Bacteroidetes* do not change in abundance after acclimation to the laboratory environment, irrespective of the water temperature.

Despite the picture of the microbiome at a higher taxonomic level, changes in the gill microbiome composition were observed at genus level. In the present study, an increase in the abundance of the genus *Mycoplasma* (phylum *Tenericutes*)

Table 3. Alpha diversity (number of observed amplicon sequence variants (ASVs) ± s.e.) of gill microbiota in oysters challenged with OsHV-1 and in unexposed controls maintained at different temperatures.

Temperature treatment	Number of observed ASVs (mean ± s.e.)		
	Pre-challenge	OsHV-1 challenged	Negative control
21°C static	323.3 ± 29.4 (n = 4)	603.9 ± 103.0 (n = 8)	251.8 ± 7.1 (n = 4)
22°C static	312 ± 40.8 (n = 10)	418.5 ± 24.2 (n = 8)	424 ± 90.1 (n = 4)
26°C static	340 ± 55.3 (n = 7)	309 ± 38.3 (n = 8)	299 ± 78.2 (n = 3)
22/26°C dynamic	435.6 ± 68.5 (n = 8)	516 ± 51.4 (n = 8)	357.8 ± 27.2 (n = 4)

was observed in the 21°C static treatment group. *Mycoplasma* has been reported to naturally dominate the oyster microbiome in warmer seawater temperatures (King *et al.* 2012). Wegner *et al.* (2013) observed an increase in the *Mycoplasma* fraction in the gill microbiome after subjecting the oysters to a disturbance treatment which mainly involved an increase of temperature (2–26°C) along with transfer to the laboratory environment and absence of feed. *Mycoplasma* species represent a temperature-sensitive component of oyster microbiota and may selectively proliferate at higher temperatures (Wegner *et al.* 2013). *Mycoplasma* have been associated with disease in shellfish (Paillard *et al.* 2004), in other aquatic invertebrates (Krol *et al.* 1991; Azevedo 1993) and in fish (Kirchhoff *et al.* 1987). Thus, the increase of *Mycoplasma* at warmer seawater temperatures may indicate an opportunistic pathogenic role of *Mycoplasma*. However, an increase in the *Mycoplasmae* fraction was not noted at 26°C in the present study.

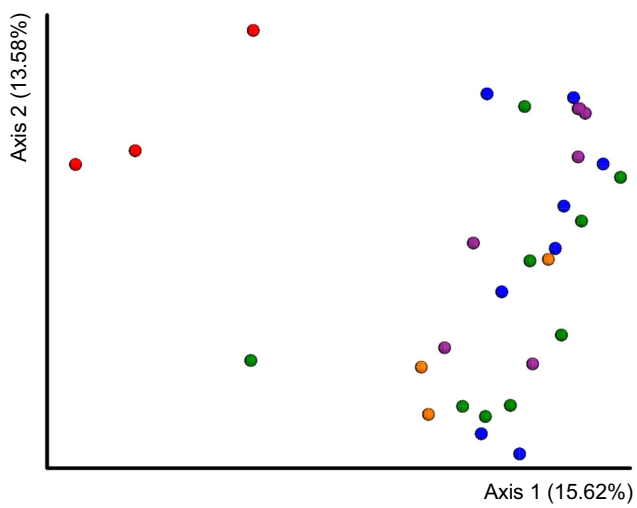

Fig. 4. Principal coordinate plot based on Bray–Curtis distances between the gill microbiome of Pacific oysters before and after acclimation to different seawater temperatures. The gill microbiota of oysters before acclimation (red; 20°C) were distinct from those after acclimation to temperatures constant 21°C (orange), 22°C (green), 26°C (purple) and diurnal fluctuation to 22/26°C (blue; $P < 0.05$).

The abundance of genus *Arcobacter* (phylum *Proteobacteria*) decreased in the gill microbiome of oysters during acclimation to different temperature profiles in this study. *Arcobacter* are microaerophilic and may respond favorably to the periodic valve closing in oysters in intertidal environments (Vandamme and De Ley 1991). As the oysters were constantly immersed in water in this study, reduced exposure to a microaerophilic environment may have limited multiplication of *Arcobacter*. The increase of phylum *Spirochaetes* in the present study was in line with the results of previous studies in other oyster species. Seasonal temperature variation in seawater has been related to variations in the microbiome composition in the gill of spondylus oysters, which included an increase of phylum *Spirochaetes* throughout summer, with temperatures ranging from 23°C to 31°C (Roterman *et al.* 2015).

The OsHV-1 challenge of the present study resulted in mortality in oysters at each temperature. A graded response was observed, with mortality lowering with temperature down to 21°C. The incubation period was also longer by 1 day at the lowest water temperature. The highest total cumulative mortality in oysters was observed in the 26°C static treatment group coupled with a higher OsHV-1 content. Mortality related to OsHV-1 usually occurs at water temperatures between 16°C and 24°C, under field conditions (Pernet *et al.* 2012; Renault *et al.* 2014; Petton *et al.* 2015; Whittington *et al.* 2019). Laboratory models have also shown mortality related to OsHV-1 at 26°C (de Kantzow *et al.* 2016; Delisle *et al.* 2018). On the basis of studies conducted in oysters in the field and in the laboratory, the OsHV-1 DNA content has a positive relationship with the seawater temperature (Petton *et al.* 2013; de Kantzow *et al.* 2016). Following a standard OsHV-1 exposure, the quantity of OsHV-1 DNA in moribund oysters at 26°C has been shown to be approximately six-fold greater than that at 18°C and 1.2-fold greater than that at 22°C (de Kantzow *et al.* 2016). A recent laboratory study conducted by Delisle *et al.* (2018) also showed a higher OsHV-1 DNA content in oysters at 26°C than in those at 21°C and 29°C. Apart from increasing the OsHV-1 load, the oyster microbiome was disturbed in elevated seawater temperatures, favouring shifts in the composition towards pathogen-dominated communities (Lokmer and Wegner 2015; Le Roux *et al.* 2016).

Table 4. Average relative abundance of the dominant genera in the gill microbiota of oysters before and after the temperature treatments and 48 h post-OsHV-1 challenge.

Treatment group	Average relative abundance (%)					
	Before	21°C_static	22°C _static	26°C _static	22/26°C _dynamic	Significance
After acclimation						
Vibrio	0.11	0.26	0.62	1.50	0.41	$P > 0.05$
Polaribacter	0.02	0.16	0.23	0.04	0.06	$P > 0.05$
Mycoplasma	0.00	5.91[A]	0.86	0.24	0.48	$P = 0.00$
Arcobacter	1.18[B]	0.03	0.08	0.03	0.04	$P = 0.01$
After OsHV-1 challenge						
Vibrio	–	2.11	5.50	10.89	2.70	$P = 0.00$
Polaribacter	–	1.97	1.99	0.19	1.08	$P = 0.00$
Mycoplasma	–	0.20[C]	0.06	0.03	0.12	$P = 0.00$
Arcobacter	–	0.01	0.02	0.04	0.04	$P > 0.05$
Negative control						
Vibrio	–	1.72	1.96	6.35	3.58	$P > 0.05$
Polaribacter	–	0.35	1.71	0.28	0.21	$P > 0.05$
Mycoplasma	–	0.21[C]	0.03	0.04	0.11	$P = 0.00$
Arcobacter	–	1.55	0.01	0.01	0.37	$P = 0.01$

Microbiomes were analysed from gill tissue samples ($n = 6$–10) collected from each treatment group.
[A]The genus *Mycoplasma* increased in abundance after acclimating to 21°C static treatment and this abundance was higher than that in oysters acclimated to 22°C and 26°C static treatments ($P < 0.05$).
[B]The genus *Arcobacter* decreased in abundance after acclimating to all temperature treatments ($P < 0.05$).
[C]The genus *Mycoplasma* decreased in abundance after OsHV-1 challenge and in negative control oysters maintained at 21°C static treatment ($P < 0.05$).

Concurrent with differences in the quantity of OsHV-1 content and mortality in different temperature profiles, increased abundance of the *Vibrio* fraction was observed in oysters maintained in all the temperature profiles tested in this study. These increases were not seen in the negative control counterparts, indicating an association of the OsHV-1 infection with the increase in the abundance of *Vibrio*. The higher increase of *Vibrio* observed in OsHV-1-challenged oysters at 26°C indicates a role of increased seawater temperature in facilitating the increase in *Vibrio* abundance. Moreover, the concurrent increase in *Vibrio* abundance with the OsHV-1 also strengthens the potential role of *Vibrio* in the proposed polymicrobial pathogenesis in OsHV-1-associated oyster mortality events. The complex aetiology of disease caused by OsHV-1 was recently investigated by de Lorgeril *et al.* (2018). They showed that a primary infection with OsHV-1 in oyster haemocytes resulted in bacteraemia with opportunistic bacteria. The same study demonstrated that *Vibrio* was one of the two main genera associated with this opportunistic role, in completing the disease pathogenesis initiated by OsHV-1. Increases in the abundances of the genera *Bacteroides*, *Vibrio* and *Arcobacter* were reported in natural mortality events of Pacific oysters that were associated with OsHV-1, *Vibrio aestuarianus* and *Vibrio harveyi* (King *et al.* 2019*a*; Lasa *et al.* 2019). With a comparative analysis, Lasa *et al.* (2019) showed that both *Vibrio* and *Arcobacter* increased in abundance in oysters infected with *Vibrio aestuarianus,* whereas the only the *Vibrio* fraction increased in OsHV-1 infections. The emerging view of the polymicrobial pathogenesis in Pacific oyster mortality (in both the presence and absence of OsHV-1) has been strengthened by an array of studies that analysed oyster microbiota both from natural field outbreaks (King *et al.* 2019*a*; Lasa *et al.* 2019) and from laboratory infection models (Petton *et al.* 2019; Pathirana *et al.* 2019*b*).

In the present study, increase in the *Vibrio* fraction arising from the microbiome was only seen after the OsHV-1 challenge and not after the temperature acclimation phase. This was consistent with previous observations of an immunosuppressive effect of OsHV-1 (de Lorgeril *et al.* 2018). The OsHV-1 challenge resulted in an increased abundance of *Vibrio* and a mortality rate of 84.4% at 26°C. Moreover, this increase was not seen in the negative control oysters. Gill tissue being a predilection site for OsHV-1, this increase in the abundance of *Vibrio* in the gill microbiome is in line with the recent findings of de Lorgeril *et al.* (2018). With a histopathological analysis, these authors showed that bacteria accumulated in the gill tissue at the onset of the viral infection, which was supported by an increase of *Vibrio* counts. Lokmer *et al.* (2016*a*) demonstrated a higher *Vibrio* load in solid tissues of oysters during mortality

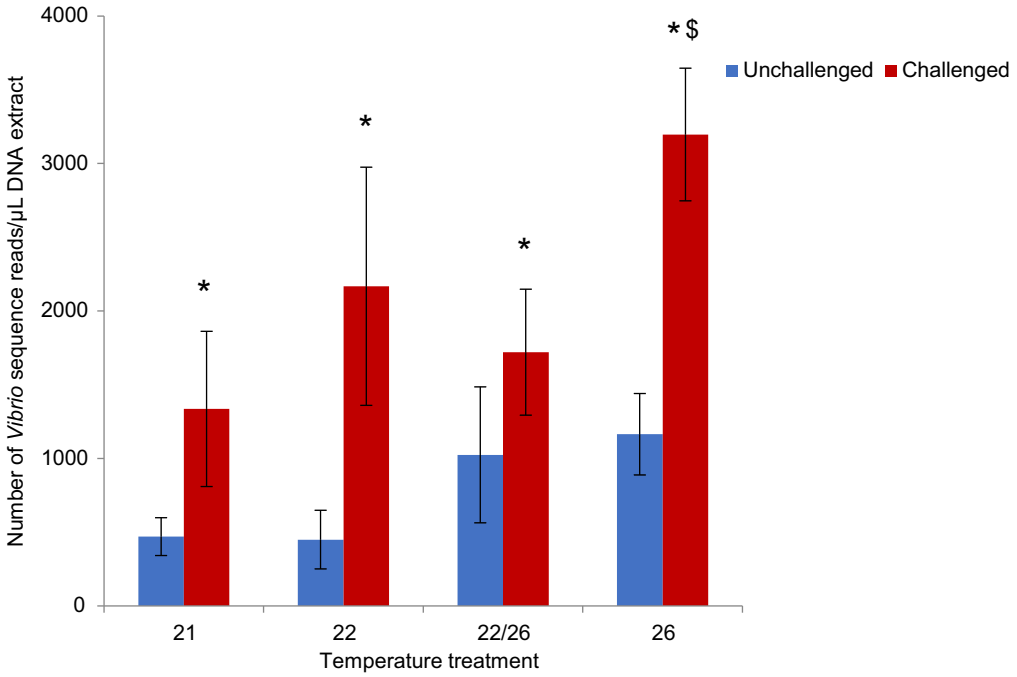

Fig. 5. Absolute abundance (number of DNA sequence reads) of the genus *Vibrio* associated with the gill of oysters challenged with OsHV-1 and maintained at different temperatures. *Higher abundance of *Vibrio* in oysters challenged with OsHV-1 than in unchallenged oysters at the same temperature and sampled at the same time ($P < 0.05$); $^{\$}$Higher abundance of *Vibrio* in OsHV-1-challenged oysters at 26°C than in those at other temperatures in the study ($P < 0.05$).

that occurred after translocation into a new environment. In addition to OsHV-1, several members of the genus *Vibrio* are considered to play a primary role in oyster mortality outbreaks (Vezzulli *et al.* 2015). Natural mortality events due to *Vibrio* infections are mostly reported in adult oysters (Travers *et al.* 2015). Although we could not isolate and test the pathogenicity of *Vibrio* in the present study, the higher quantity of *Vibrio* reported at 26°C may have played a secondary opportunistic role in oyster mortality. Fluctuating water temperatures in the range 22–26°C did not alter OsHV-1 associated mortality in this study compared with 26°C, but there was a higher total bacteria quantity. Other environmental factors such as tidal emersion may interact with changing temperatures in the natural estuarine environment to produce a different picture.

The present study did not observe any increase in *Arcobacter* in the gill microbiome after the OsHV-1 challenge. Lasa *et al.* (2019) also reported an increase in *Vibrio* alone in OsHV-1 infections, while abundances of both *Vibrio* and *Arcobacter* species increased in *Vibrio aestuarianus* infections. At higher taxonomic levels, the abundance of phylum *Proteobacteria* and phylum *Bacteroidetes* increased in the gill microbiome after OsHV-1 challenge.

This study observed a lower alpha diversity in the gill of oysters maintained at a constant 26°C, a temperature above the preferred thermal range of Pacific oysters (Bourlès *et al.* 2009; Petton *et al.* 2013). Dysbiosis (loss of bacterial diversity and proliferation of few operational taxonomic units) has repeatedly been associated with impaired oyster health (Garnier *et al.* 2007; Green and Barnes 2010; Lokmer and Wegner 2015; Pathirana *et al.* 2019*b*). The shorter clinical course (5 days as compared with 7 and 8 days) for oysters maintained at 26°C may have been influenced by an existing state of dysbiosis.

The dynamic temperature treatment (22/26°C) employed in this study did not result in marked changes in oyster mortality or in the oyster microbiome compared with similar static temperatures. Although the required temperature changes were reached in seawater, the time taken to reach the peak temperature was longer and the experimental design did not allow the peak temperature to remain at that level for a long period. While this is reflective of water temperature, oysters in an intertidal environment can be exposed to much higher and more rapid changes in temperature when exposed to air, at a time when the valves are shut, and this might have a greater impact on the microbiome.

Conclusions

The water temperature profiles provided in the present laboratory aquaria did not affect the quantity of bacteria

associated with oysters but did alter the bacterial community composition. The degree and nature of these changes varied with the water temperature profile and reflected differences among bacterial genera. The opportunistic role of *Vibrio* in OsHV-1-associated oyster mortality appeared to be further facilitated by the seawater temperature. Higher oyster mortality was not only associated with a higher water temperature and a higher OsHV-1 load but was also associated with the highest *Vibrio* concentration. Except for the increase in total bacterial quantity after OsHV-1 challenge, the diurnal temperature fluctuations between 22°C and 26°C did not decrease the stability of the oyster microbiome in this laboratory setting, compared with constant temperatures.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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