Characterisation of the Pacific oyster microbiome during a summer mortality event

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Key words: *Crassostrea gigas*, mortality, oyster, microbiome, Summer mortality

Abstract

The Pacific oyster, *Crassostrea gigas*, is a key commercial species that is cultivated globally. In recent years, disease outbreaks have heavily impacted *C. gigas* stocks worldwide, with many losses incurred during summer. A number of infectious agents have been associated with these summer mortality events, including viruses (particularly Ostreid herpesvirus 1, OsHV-1) and bacteria, however cases where no known aetiological agent can be identified are common. In this study, we examined the microbiome of disease-affected and disease-unaffected *C. gigas* during a 2013-2014 summer mortality event in Port Stephens (Australia) where known oyster pathogens including OsHV-1 were not detected. The adductor muscle microbiomes of 70 *C. gigas* samples across 12 study sites in the Port Stephens estuary were characterised using 16S rRNA (V1-V3 region) amplicon sequencing, with the aim of comparing the influence of spatial location and disease state on the oyster microbiome. Spatial location was found to be a significant determinant of the disease-affected oyster microbiome. Furthermore, microbiome comparisons between disease states, identified a significant increase in rare operational taxonomic units (OTUs) belonging to *Vibrio harveyi* and an unidentified member of the *Vibrio* genus in the disease-affected microbiome. This is indicative of a potential role of *Vibrio* species in oyster disease and supportive of previous culture-based examination of this mortality event.

Introduction

The Pacific oyster, *Crassostrea gigas*, is the most heavily cultivated oyster species globally. However, in recent years, production of *C. gigas* has been compromised by widespread and recurrent mortality events [1-6]. Mortalities frequently occur during the summer months, with "summer mortality" often used as an umbrella term to encompass mortalities resulting from viral and/or bacterial infection overlaid with (or precipitated by) environmental stressors [1, 7, 8].

Outbreaks of viral infections have largely been attributed to an infection by the ostreid herpesvirus (OshV-1) or its micro-variant (OsHV-1 μvar), which affects oyster larvae, spat or juveniles [1, 6, 9]. These OsHV-1 infections have been implicated as the causative agent of Pacific Oyster Mortality Syndrome (POMS), particularly under elevated water temperatures [10-12].

In some instances of *C. gigas* summer mortality, bacteria may also play a role with several members of the *Vibrio* genus implicated as potential disease-causing agents [7, 13, 14]. These *Vibrio* infections typically target the larval and spat life stages but can also be induced in adult oysters through experimental injection challenges [7, 13]. Infections with both *Vibrio* species and OsHV-1 have been previously recorded [15] with *Vibrio* species potentially acting synergistically with OsHV-1 [16].

It is notable, however, that in many instances of summer mortality, no clear aetiological agent has been identified [7, 17]. For these summer mortality events (and in fact for many other oyster diseases), a number of different environmental and physiological factors, including temperature, nutrient concentrations, chlorophyll *a* levels, turbidity, salinity, oyster growth rate

and reproductive effort have been implicated as triggers for mortality events [4, 8, 18-21]. However, in most cases no single clear determinative factor(s) has been found. There is also evidence that the severity of summer mortality events is influenced by the host's genetic background and this is being exploited for disease management by breeding resistant genetic lines [22-25].

Another potential factor in disease events, involves the role of the oyster microbiome. Previous studies have shown the *C. gigas* microbiome to be dynamic and responsive to external factors [26, 27], with the microbial community responding to heat, translocation, bacterial infection and antibiotic stressors [26-28]. The microbiome is also influenced by host factors, such as the genetics of the individual oyster [26] and the oyster life stage [29, 30].

The role of the oyster microbiome in disease progression is an area gaining interest. Previous work has shown that while infection with OsHV-1 plays an important role in POMS, oysters pre-treated with antibiotics do not succumb to mortality, indicating that the oysters' microbiome is a factor in disease progression [16]. Mortality was also correlated with low species evenness of hemolymph microbiome before translocation stress allowing *Vibrio* species to invade oyster tissues [28]. In addition, the resident *Vibrio* community has been observed to be replaced by virulent strains before a summer mortality disease outbreak [31].

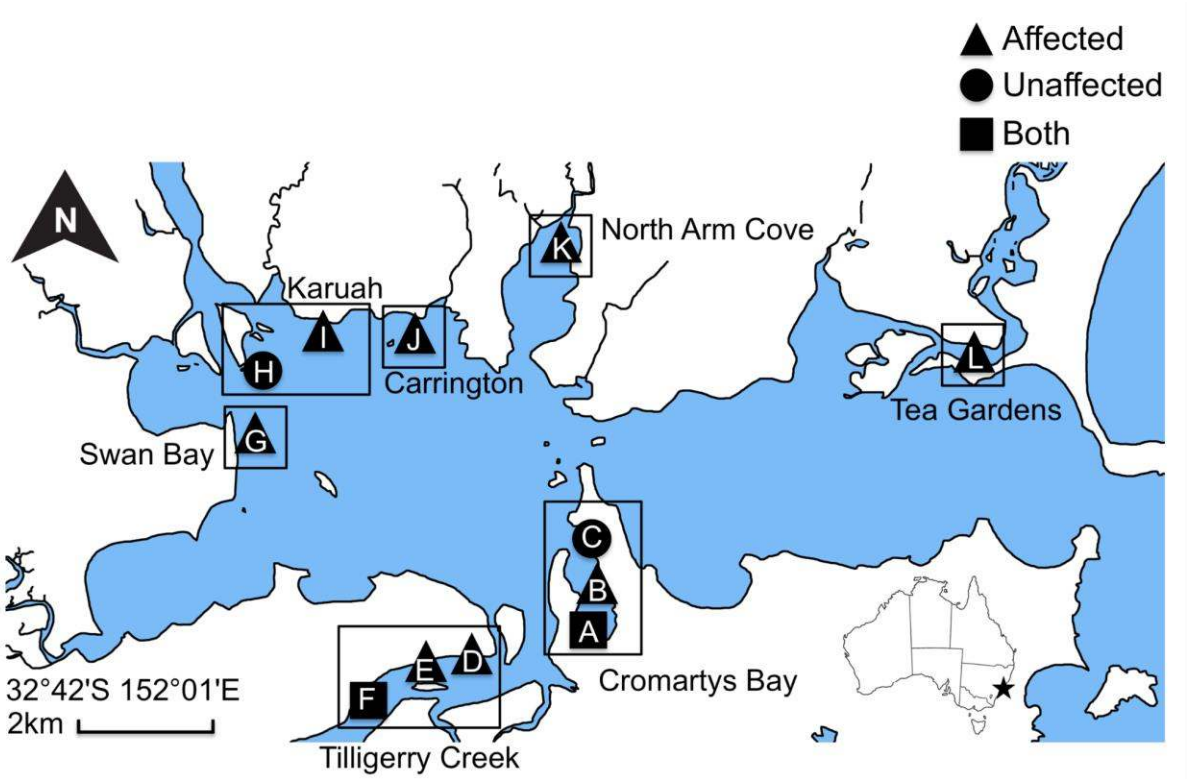
In the summer of 2013 to 2014, a sudden mortality event occurred in the Port Stephens estuary, New South Wales, Australia. The New South Wales Department of Primary Industries (NSW DPI) obtained oysters as a part of a structured survey to identify potential aetiolgical agents involved in this mortality event [17]. All oysters were found to be negative for the presence of OsHV-1 and OsHV-1 µvar and other known oyster pathogens [17]. Bacterial cultivation work

identified numerous *Vibrio* species (*V. crassostreae*, *V. splendidus*, *V. harveyi* and *V. alginolyticus*) as being dominant in different sites, although no single clear dominant bacterium was consistently seen across the estuary [17]. Environmental data collected at the time indicated that sudden decreases in salinity due to rainfall and high temperature (>20°C) were likely contributors to the mortality event [17]. As no clear aetiological agent was found during the structured survey and to further explore this mortality event, we aimed to compare the microbiome of *C. gigas* oysters from disease-affected and disease-unaffected sites and to explore whether the oyster microbiome was influenced by spatial variation.

Methods

Oyster study sites and cultivation

Port Stephens is a tide-dominated estuary [32], located on the east coast of New South Wales (NSW), Australia (32°41'53.9"S 152°01'26.3"E; Figure 1). During a major mortality event that occurred in the austral summer of 2014 (January 6-13) *C. gigas* oysters were collected from leases where mortality was recorded. These leases were spread over seven localities within the Port Stephens estuary, of which five (Swan Bay, Karuah, Carrington, North Arm Cove and Tea Gardens) were situated in the northern part of the estuary and two (Cromartys Bay and Tilligerry Creek) in the southern part (Figure 1). Among these seven localities, there were twelve sampling sites. Of these, ten sites were cultivated for commercial purposes and affected by the mortality outbreak (will be referred to as disease-affected) whereas two sites (one in Cromartys Bay and one in Karuah) were disease-unaffected wild oysters (disease-unaffected will be referred to as unaffected). The southern part of the Port Stephens estuary contains the bulk of the urban population [33], with only Tea Gardens in the far north eastern part of the estuary with a comparable population size [33], the southern sites also have a strong agricultural and mining industrial presence, particularly near Tilligerry Creek [34].



126

127 **Fig. 1.** Sampling locations and sites across the Port Stephens estuary. Areas with more than
128 one sampling site are designated as localities and are contained within boxes. Sites are
129 numbered A through to L, which corresponds to the site numbers in Table 1.

130

131 Site A in Cromartys Bay contained two stocks that were either affected or unaffected by the
132 mortality event (Figure 1). Site F in Tilligerry creek had two stocks with different final
133 mortalities (10% and 99%). Due to the low mortality for this stock (10%) and the significantly
134 different microbial assemblage (One-way ANOSIM using five samples per stock: $p = 0.0067$;
135 $R = 0.61$ with 9999 permutations) to the 99% mortality stock, it was treated as an unaffected
136 stock. The oyster cultivation conditions at each site (where known) are summarised in Table 1.
137 Diploid oysters were cultivated at all sites, with the exception of one site in Tilligerry creek,
138 where triploid oysters were cultivated. Oysters were predominately grown in trays except for
139 those in North Arm Cove and Karuah (disease-affected site), which were grown in long line

baskets. Cultivated oysters were sourced from a Tasmanian hatchery, a Port Stephens-based hatchery or, wild-caught oyster seed or were wild non-cultivated oysters.

Table 1 – The oyster stock source, ploidy status and cultivation method (where known) for oysters at all study sites

Locality	**Site number**	**Ploidy**	**Stock source**	**Cultivation method**
Cromartys Bay	Site A	Diploid	Port Stephens hatchery	Trays
Cromartys Bay	Site B	Diploid	Tasmanian hatchery	Trays
Cromartys Bay	Site C	Diploid	Wild	Wild[B]
Tilligerry Creek	Site D	Diploid	Port Stephens hatchery	Trays
Tilligerry Creek	Site E	Diploid	[NA]	Trays
Tilligerry Creek	Site F	Triploid	Tasmanian hatchery	[NA]
Swan Bay	Site G	Diploid	Port Stephens nursery	[NA]
Karuah	Site H	Diploid	Wild	Wild[NB]
Karuah	Site I	Diploid	Hatchery[NA]	Long line baskets
Carrington	Site J	Diploid	Wild caught	[NA]
North Arm Cove	Site K	Diploid	Wild caught	Long line baskets
Tea Gardens	Site L	Diploid	Wild caught	[NA]

[NA]Information not supplied by the oyster farmer; [B]Wild oysters were not grown on a commercial lease

Oyster sample processing and DNA extraction

Juvenile oysters were collected from each site and transported back to the laboratory in iced containers as previously described [17], where they were stored at -80 °C prior to analysis. Five samples from each sampling site were thawed and scrubbed with a hard bristled brush under running water to remove any remaining mud and debris on the outer shell. Samples were then shucked using sterile shucking knives and immediately placed into sterile petri dishes. Approximately 25 mg of adductor muscle tissue was dissected and removed using sterile scalpel blades. Hemolymph collected *via* aspiration of the adductor muscle sinuses is frequently used to examine the bacterial population circulating within oysters [27, 28, 35, 36] however, the use of frozen oysters in this study precluded aspiration of the hemolymph via syringe. Therefore, in line with the approaches used in several previous studies [27, 28, 35] aseptically dissected adductor muscle tissue containing hemolymph sinuses [37] was used to capture the fluid contained within.

DNA was extracted from the dissected adductor muscle using the Qiagen DNeasy blood and tissue kit (catalogue: 69506), as per the manufacturer's instructions. Extracted DNA was then amplified using PCR targeting the ribosomal 16S rRNA V1-V3 region using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') primer pair [38, 39]. The PCR cycling conditions were as follows: 94°C for two minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Amplicons were sequenced using the Illumina MiSeq platform (version 3, 2 x 300bp) at the Ramaciotti Centre for Genomics (University of

New South Wales, Sydney, Australia). Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) with the study accession number (SRP139423) under Bioproject number PRJNA449563.

Data analysis

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME version 1.9.1) pipeline [40]. Briefly, paired-ended DNA sequences were joined with join_paired_ends.py, OTUs were defined at 97% sequence identity using UCLUST [41] using open-reference picking, and taxonomy was assigned against the Greengenes database (version 13/08/2013) [42] using the RDP classifier [43]. Chimeric sequences were then identified using ChimeraSlayer. Mitochondrial, chloroplast and chimeric sequences were filtered out of the dataset. Remaining sequences were rarefied to allow for even coverage across all samples. Relative abundance per sample was calculated and those OTUs with a relative abundance below 0.1% were filtered from the dataset. Alpha diversity indices, including Shannons Index, Chao1 and Shannons Index/log (observed species) were used to calculate species diversity, species richness and species evenness respectively, using QIIME [40].

Statistical analyses

Taxonomic data was compared at the OTU level, with OTUs assigned taxonomy down to the finest resolution possible. Comparisons of alpha diversity were performed with a one-way ANOVA, with homogeneity of variance confirmed using Levene's test for homogeneity of variance. All beta-diversity comparisons were performed with a Bray-Curtis dissimilarity index. To compare beta diversity from different locations and different disease states, relative abundance was first normalised (Square root (x)) and used with a non-metric multidimensional scaling (nMDS) analysis. To determine the statistical significance of apparent patterns

identified by nMDS a one-way ANOSIM with 9999 permutations was used. To identify which OTUs contribute to the greatest differences between locations and/or disease states, SIMPER analysis was used. To determine whether specific OTUs (such as those OTUs with the highest summed abundance across all samples, and those OTUs identified as determinants of difference between samples using SIMPER) were significantly different between disease states, a Kruskal-Wallis ANOVA was used. All of these analyses were carried out using PAST [44]. To determine whether an OTU was significantly elevated in a particular disease state at Site A, the group_significance.py script using the default analysis (Kruskal Wallis ANOVA) was used in QIIME.

Results

Sequence read depth and rarification

Using adductor muscle as the tissue source, a total of 9, 692, 231 raw reads were generated from the sequencing run. Of those, the minimum read depth was 29, 753 reads with a maximum of 356, 708 reads, and a median of 127, 006 reads. ChimeraSlayer identified 17, 730 reads as chimeras (0.2% of the dataset), and were subsequently removed. Sequences were rarefied to 29, 700 reads per sample to remove the effect of sampling effects upon analysis.

Replicates of C. gigas microbiomes show large within-site heterogeneity

We observed a high level of within-site variation in the composition of the *C. gigas* microbiome (Figure 2). For instance, unaffected oyster microbiomes from Site A (Table 1), exhibited low similarity (defined by a low median) between replicates (Median ± Standard Deviation; 0.13 ± 0.10), while disease-affected replicates in Tilligerry creek (Site D; 98% mortality) had the lowest variability (defined by a low standard deviation) between individuals (0.27 ± 0.05). At Cromartys Bay site A, disease-affected oyster microbiomes had significantly less inter-oyster

variability than the unaffected oysters (Kruskal-Wallis ANOVA, p = 0.005; 0.33 ± 0.09 and 0.13 ± 0.10 respectively; Supplementary Table 1), this was also observed at Site F in Tilligerry Creek (p = 0.007; disease-affected 0.36 ± 0.097 and unaffected 0.21 ± 0.075). There was no significant difference when comparing variation between those oyster microbiomes in the north versus the south (p = 0.29). However, disease-affected microbiomes in the north had significantly less similarity between samples than those in the south (p = 0.009; 0.28 ± 0.089 and 0.34 ± 0.149 respectively). Differences in the local environment could potentially explain this difference in similarity, as the southern part of the estuary is largely urbanised and has a strong agricultural and mining presence [33].

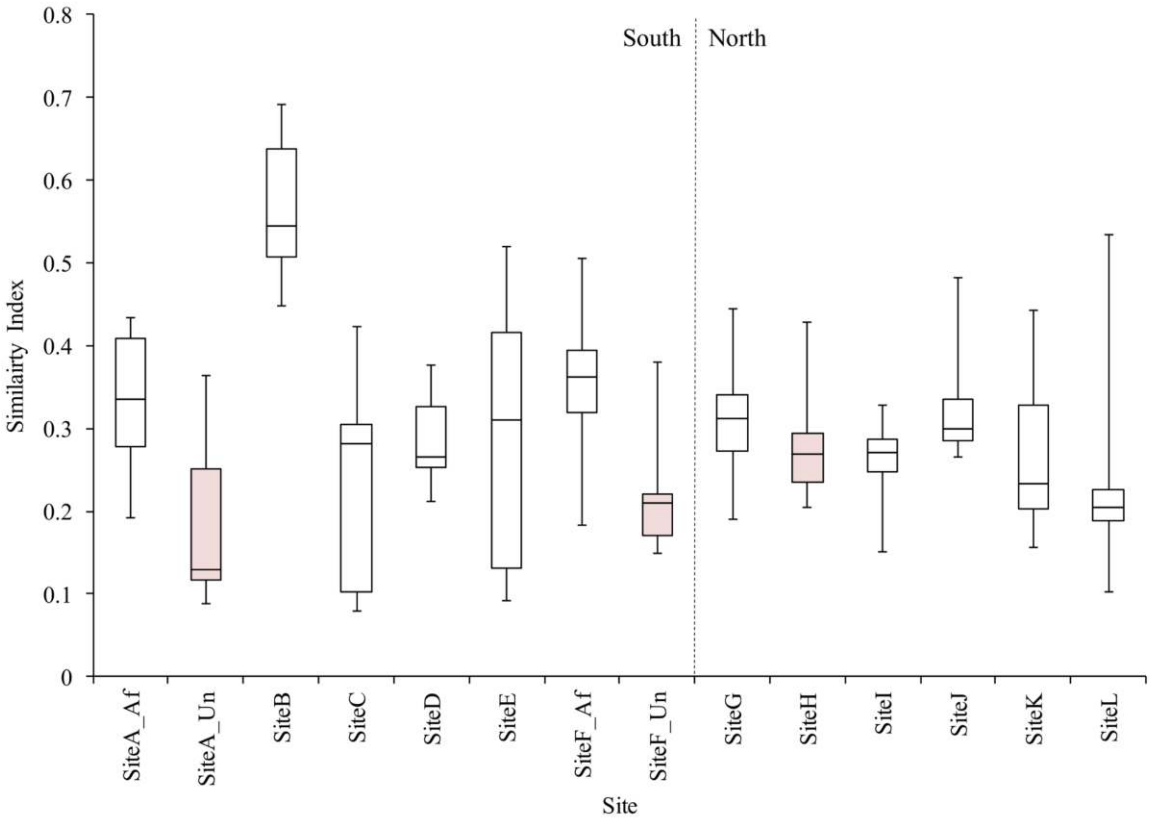
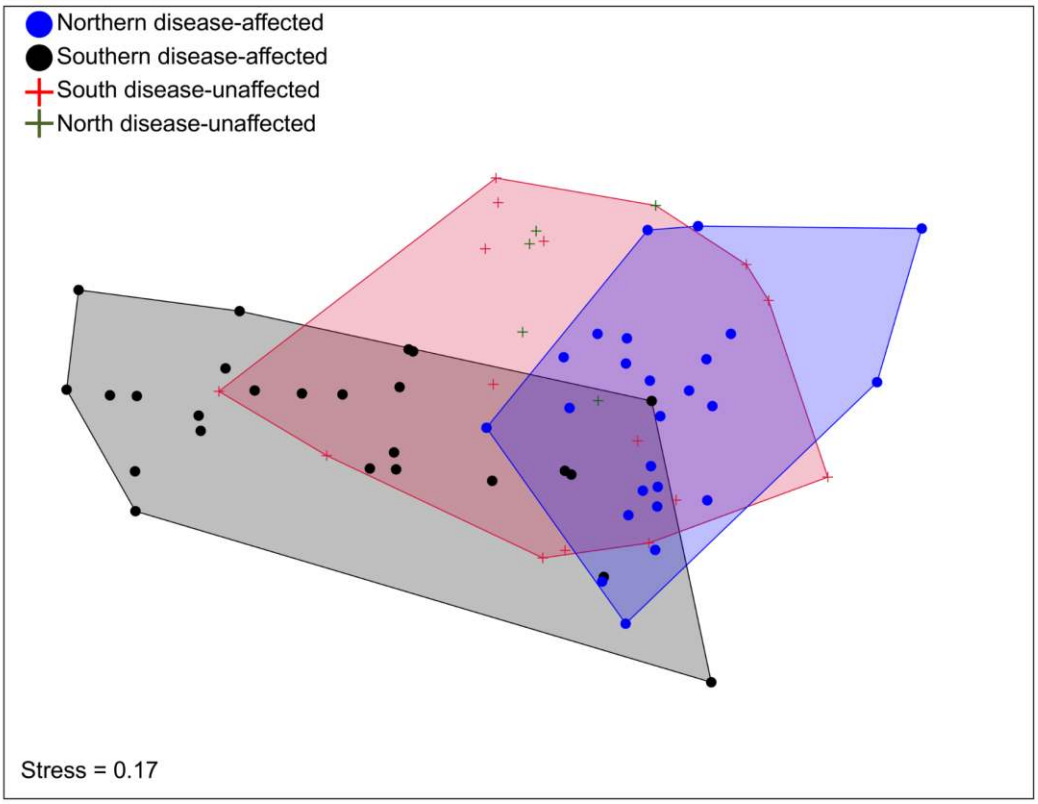


Fig. 2. Box and whisker plot of similarity indices calculated with a Bray-Curtis dissimilarity index. Each site code corresponds to those values listed in Table 1. As sites A and F contained both disease-affected and unaffected oyster stocks, these sites listed with the suffix '_Af" are

disease-affected stocks, while those with '_Un' are unaffected stocks. Each site has 5 replicate oysters. Northern and southern samples are separated by the black vertical line. Disease-unaffected sites are shaded red.

C. gigas microbiomes from Port Stephens mortality event separate both spatially and in accordance with disease state

We sought to examine whether the microbial assemblage of oyster microbiomes were influenced by disease-state, spatial location and stock source. The *C. gigas* microbiome composition significantly differed according to spatial location and disease state (Figure 3). Oyster samples taken from sites experiencing no mortalities had a significantly different microbiome composition than samples from disease-affected sites in the north ($p = 0.0002$; $R = 0.26$) and south ($p = 0.0001$; $R = 0.21$). In addition, oysters collected from sites exhibiting high mortalities within the southern region of the estuary exhibited a microbiome composition that differed significantly from the disease-affected oysters in the northern region ($p = 0.0001$; $R = 0.48$; Figure 3), with species diversity ($F_{(1, 48)} = 4.16$; $p = 0.047$;) and richness ($F_{(1, 48)} = 14.15$; $p = 0.00046$) also differing between the northern and southern regions of the estuary, while species evenness did not differ between regions ($F_{(1, 48)} = 1.07$; $p = 0.31$) (Supplementary Table 1). Disease-affected hatchery sourced oysters also had a significantly different microbial assemblage to disease-affected wild sourced oysters ($p = 0.0052$; $R = 0.19$).



254

255 **Fig. 3.** nMDS plot showing spatial and disease state separation. Samples separate spatially
256 based on region, as well as by their disease state. Axes 1 and 2 are plotted.

257

258 As we observed a significant difference between the disease-affected and disease-unaffected
259 microbiome composition, we sought to examine persistent ('core') bacteria unique to these
260 disease-states and across the estuary as a whole (Supplementary Table 2). We identified 24
261 core members, of which 1 core OTU, an unidentified member of the *Mollicutes* class (OTU
262 89399), was unique to disease-affected microbiomes, while 8 OTUs were unique to disease-
263 unaffected microbiomes (Supplementary Table 2). BLASTing the representative sequence for
264 OTU 89399 against the NCBI database identified it as an unculturered bacterium previously
265 observed in *C. gigas* and another oyster species, *Saccostrea glomerata* (both BLAST hits were
266 E-value: 0, Identity: 99%) [45, 46]. We then sought to examine which OTUs were driving the
267 microbiome composition difference between disease states, and between spatial location.

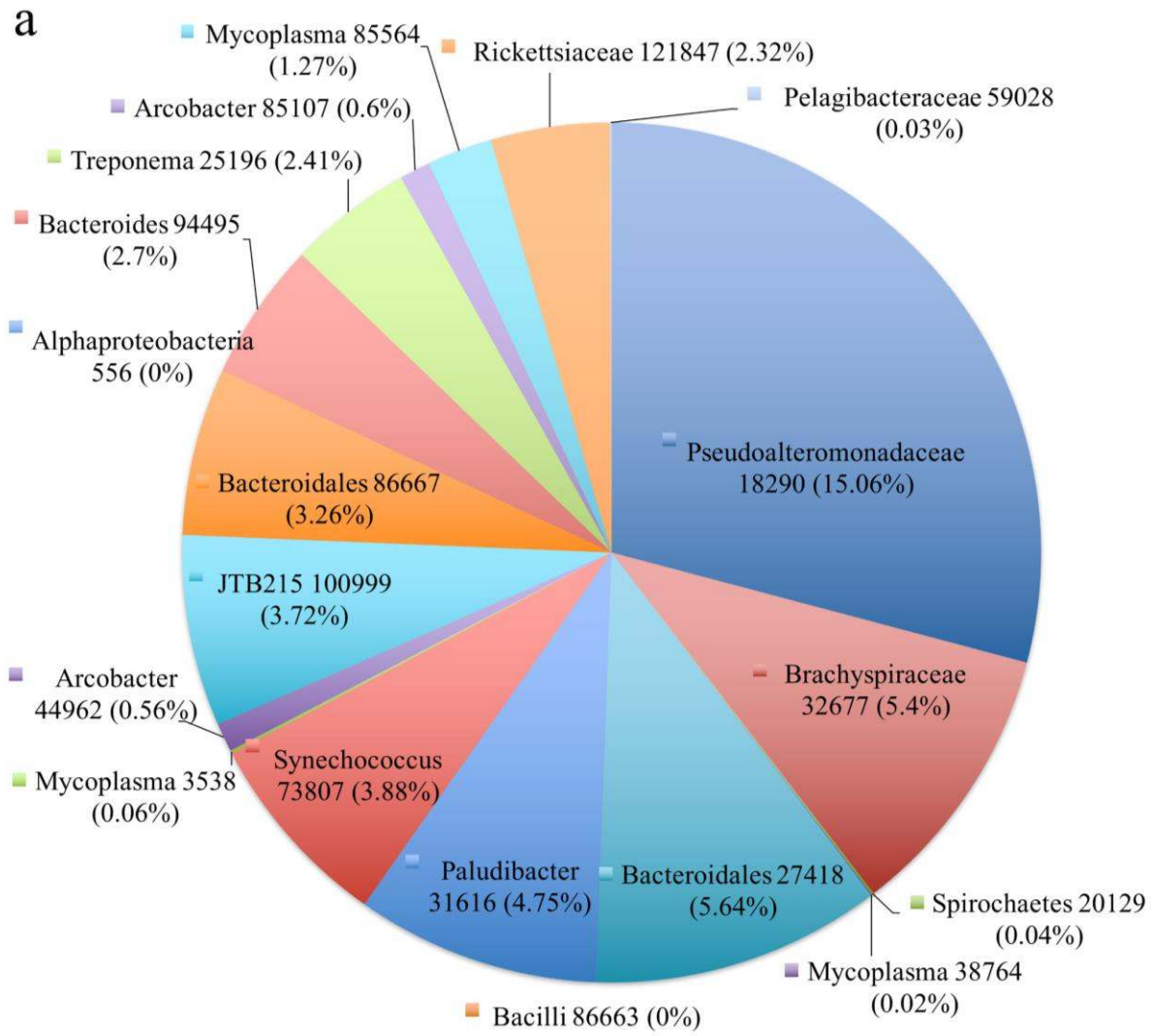
According to SIMPER analysis, the microbiomes of all unaffected samples (20 samples) were found to be 86.1% dissimilar to disease-affected samples in the south (25 samples) and 80.1% dissimilar to disease-affected samples in the north (25 samples; Supplementary Table 3).

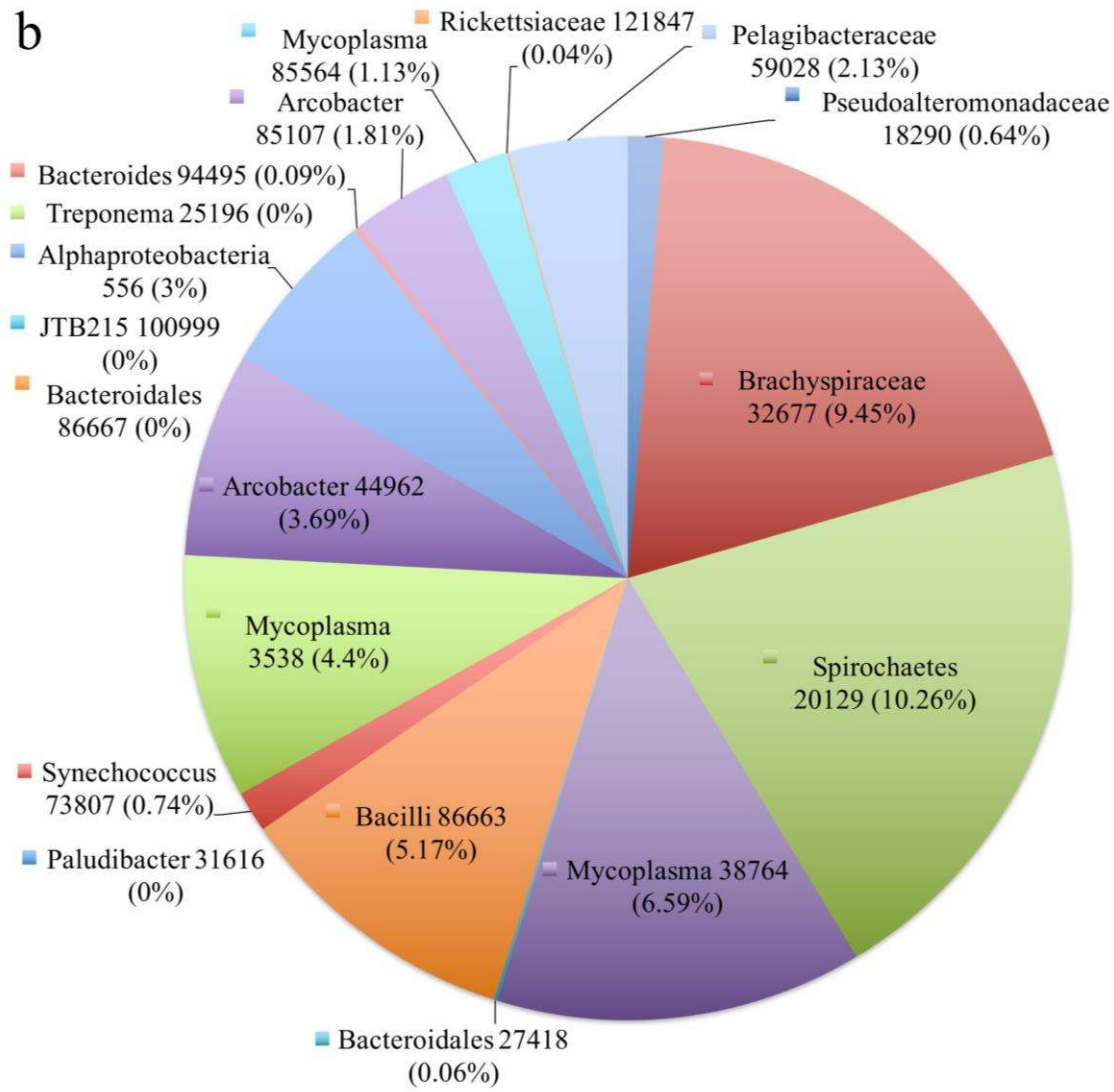
Due to the spatial separation of oyster microbial communities between the northern and southern regions of the estuary, patterns in the relative abundance of dominant OTUs within each region were examined separately to determine whether these OTUs were associated with disease-affected or unaffected oysters. In the northern region, the top five dominant OTUs from all sites belonged to the *Brachyspiraceae* family (OTU 32677), *Mycoplasma* genus (OTU 38764), *Mycoplasma* genus (OTU 3538), *Mollicutes* class (OTU 89399) and the *Alphaproteobacteria* class (OTU 556), with these OTUs representing 28.8%, 9.3%, 3.2%, 3.2% and 2.7% of the total community respectively. OTUs assigned to the *Mycoplasma* genus (OTU 38764), *Mycoplasma* genus (OTU 3538) and the *Mollicutes* class (OTU 89399) were more abundant in disease-affected samples ($p = 0.039$; $p = 0.044$ and $p = 0.0097$ respectively), while the relative abundance of an unidentified member of the *Alphaproteobacteria* class (OTU 556) and *Brachyspiraceae* family (OTU 32677) was uniform across all samples ($p = 0.73$ and $p = 0.16$ respectively).

In the south, the five most dominant OTUs across all sites were members of the *Brachyspiraceae* family (OTU 32677), *Spirochaetia* class (OTU 20129), *Mycoplasma* genus (OTU 38764), *Pseudoalteromonadaceae* family (OTU 18290) and the *Alphaproteobacteria* class (OTU 556), with these OTUs representing 10.8%, 8.3%, 7.8%, 5.2% and 3.3% of sequences in the south. OTUs assigned to the *Pseudoalteromonadaceae* (OTU 18290) and *Brachyspiraceae* family (OTU 32677) were elevated in the disease-affected samples ($p = 0.48$

292 and p = 0.00055 respectively), while the remaining dominant OTUs were found in both
293 unaffected and disease-affected samples.
294
295 *Cromartys Bay C. gigas microbiomes shift in accordance with disease state*
296 One location in Cromartys Bay (Site A) contained both disease-affected (75% mortality) and
297 unaffected (0% mortality) oyster stocks (Figure 1). Due to the apparent role of spatial location
298 in shaping the microbiome, and to account for any potentially confounding external factors
299 such as cultivation method, hatchery source and unique local microenvironments, we focused
300 on Site A to examine differences in the microbiome of unaffected and disease-affected oysters.
301 While Site F in Tilligerry Creek also had oyster stocks that were disease-affected and
302 unaffected, this site was the only studied site to grow triploid oysters and information about
303 the cultivation methods used at this site are unknown (Table 1). Because ploidy status and
304 differing cultivation methods play a role in mortality outbreaks [15], Site A was selected as the
305 preferred site for comparative analysis.
306
307 All oysters from Site A were cultivated in trays from hatchery spat that were acquired from the
308 same source, thus minimising any potential differences in the microbiome caused by host
309 genetics or cultivation method. While these two sample groups displayed no difference in
310 species diversity (p = 0.46) or evenness (p = 0.84) and only a marginally significant difference
311 with species richness ($F_{(1, 8)} = 8.5$; p = 0.019), they exhibited significantly different microbiome
312 composition (p = 0.0073; R = 0.58; Figure 4; Supplementary Figure 1; Supplementary Table
313 1), and were 86.5% dissimilar to each other according to a SIMPER analysis. This dissimilarity
314 identified with a SIMPER analysis was primarily driven by an over-representation of OTUs
315 assigned to the *Pseudoalteromonadaceae* family (OTU 18290), *Bacteroidales* order (OTU
316 27418), JTB215 family (of the order *Clostridiales*; OTU 100999), *Bacteroidales* order (OTU

86667), *Paludibacter* genus (OTU 31616) and the *Bacteroides* genus (OTU 94495) in the disease-affected samples (p = 0.0090; p = 0.0082; p = 0.0053; p = 0.0053; p = 0.019; p = 0.034 respectively; Supplementary Table 4).





322

323 **Fig. 4** Average abundance of Pacific Oyster microbiomes at Cromartys bay Site A. a)
324 represents disease-affected samples, while b) shows unaffected samples. A large shift in the
325 microbiome is evident in disease-affected samples. OTUs representing less than 0.1% relative
326 abundance were filtered out of the data set and only the top 20 OTUs are displayed,
327 representing 51.7% and 49.2% of the averaged relative abundance community in a) and b)
328 respectively. Numbers in brackets on the pie chart represent the average relative abundance for
329 each pie slice.

330

To examine whether there were significant changes in the relative abundance of OTUs between disease states, we used the group_significance.py analysis with the default Kruskal-Wallis ANOVA parameters, of these, we chose OTUs assigned to the genus and species level for further examination. OTUs assigned to the *Paludibacter* genus (OTU 31616; p = 0.0070), *Bacteroides* genus (OTU 94495; p = 0.022), *Treponema* genus (OTU 25196; p = 0.0050), *Arcobacter* (OTU 4188; p = 0.011), *Vibrio harveyi* (OTU 67592; p = 0.0060) and *Vibrio* genus (OTU 122517; p = 0.015) were significantly elevated in disease-affected oysters (Table 2), while OTUs assigned to the *Pseudoalteromonas* genus (OTU 38778; p = 0.016), *Mycoplasma* genus (OTU 109572; p = 0.030), *Costertonia aggregata* (OTU 16511; p = 0.025) and the *Amphritea* genus (OTU 69264; p = 0.05) were significantly overrepresented in disease-unaffected samples (Table 2).

Table 2 – Significantly elevated OTUs (Kruskal-Wallis ANOVA; $p < 0.05$) between disease states at Site A, as well as their average abundance. OTUs assigned down to the genus or species level were chosen.

OTU	**Diseased abundance (%)**	**Healthy abundance (%)**
Bacteroides 94495	2.70	0.087
Paludibacter 31616	4.75	0
Costertonia aggregata 16511	0	0.18
Formosa crassostrea 88998	0.024	0
Tenacibaculum 125471	0	0.029
Fusibacter 119674	0.028	0
Fusobacterium 6434	0.57	0
Psychrilyobacter 42830	0.038	0

Nautella 120088	0.52	0.027
Octadecabacter antarcticus 25878	0.15	0
Desulfotalea 96648	0.069	0
Arcobacter 4188	1.70	0
Amphritea 69264	0	0.099
Pseudoalteromonas 38778	0	0.56
Pseudoalteromonas piscicida 110272	0	0.036
Vibrio harveyi 67592	0.15	0
Vibrio 122517	0.22	0
Treponema 25196	2.41	0
Mycoplasma 109572	0	0.36

346

347 In addition, examinations of those most abundant OTUs at Site A identified members of the
348 *Pseudoalteromonadaceae* family ($p = 0.0090$) and *Bacteroidales* order ($p = 0.0082$) as being
349 dominant in disease-affected samples (9.4% and 3.4% of the total community respectively)
350 while the *Spirochaetia* class, *Brachyspiraceae* family and the *Mycoplasma* genus were
351 uniformly abundant in all samples. There were also 178 OTUs and 273 OTUs exclusively
352 present in the disease-affected and unaffected group respectively, but these OTUs were
353 typically rare (less than 1% average abundance), with the exception of the *Paludibacter* genus
354 (OTU 31616; $p = 0.019$), *JTB215* family (of the order *Clostridiales*; OTU 100999, $p = 0.0053$),
355 *Bacteriodales* order (OTU 86667, $p = 0.0053$), *Treponema* genus (OTU 25196; $p = 0.054$) and
356 the *Arcobacter* genus (OTU 4188; $p = 0.0053$), which made an average relative abundance of
357 4.8%, 3.7%, 3.3%, 2.4% and 1.7% respectively in disease-affected samples and contributed to
358 1.7%, 1.7%, 1.7%, 0.9% and 1.2% of the difference between disease states respectively.

Discussion

Oyster microbiomes have large within-site heterogeneity

Oyster samples examined in this study displayed a high degree of within-site microbiome variability, which is consistent with previous work that has demonstrated substantial inter-oyster heterogeneity in microbiome composition [26]. A previous study has shown that the rare specialist community is governed by the genetics of individual oysters [26], which may be responsible for the variability between replicate oysters. However, as we do not have any information pertaining to the population structure of these oysters we cannot account for the genetic diversity between wild and hatchery sourced oysters. Despite the inter-oyster within-site oyster microbiome heterogeneity, the variability between northern and southern sites and disease state were larger. Nonetheless, the high variability between individual microbiomes may be the reason for the low power for many of the statistical tests performed here, suggesting that future studies examining oyster microbiomes might need to account for this with increased replicates.

Oyster microbiomes are influenced by spatial location

The high degree of location specific difference between diseased samples in the northern and southern regions of the Port Stephens estuary was arguably surprising given that the estuary is only approximately 5km wide. In contrast to our observations, little to no spatial heterogeneity in the composition of oyster microbiomes was observed across the Wadden Sea in Northern Europe, which spans an area of approximately 200km [35]. A previous study indicates that genetics plays a minor role in explaining the variability between individual oyster microbiomes [26], in agreement, the oyster microbiomes of hatchery sourced disease-affected oysters (primarily from southern sites) were found to be significantly different to wild sourced disease-

384 affected oyster microbiomes (primarily northern sites), therefore it is possible that genetics
385 play a small role in explaining the spatial separation of oyster microbiomes in this study.
386 However, further research is required to isolate the importance of these variables on the
387 disease-affected oyster microbiome.
388
389 *Within site comparison of microbiomes between disease states*
390 As the site at Cromartys Bay (Site A) contained oysters from both disease-affected and
391 unaffected trays, we could remove the confounding effects of spatial variation to examine the
392 within-site differences in the microbiome of disease-affected and disease-unaffected oysters.
393 At Site A, OTUs belonging to *V. harveyi* and an unidentified member of the *Vibrio* genus
394 (Table 2) were found to be significantly more abundant in disease-affected samples. This is
395 consistent with previous studies that have implicated the *Vibrio* community for their role in
396 oyster disease outbreaks [7, 31]. *C. gigas* experimentally infected with a virulent *Vibrio* strain
397 show an increase in *Vibrio* abundance in the microbiome, but the relative abundance remains
398 low, despite significant disease symptoms and shifts in the rest of the microbiome [27].
399 Similarly, a small non-significant fold increase (~1.4) of *Vibrio* spp. abundance in heat stress
400 corals is sufficient to cause large microbiome metabolic shifts [47]. Replacement of non-
401 virulent strains with virulent strains has been documented in *C. gigas* during a summer
402 mortality disease event [31] indicating that rare *Vibrio* species can disrupt the microbiome to a
403 disease susceptible state. While 16S rRNA is often unable to provide sufficient resolution to
404 observe shifts within *Vibrio* populations, we observed increases in *V. harveyi*. This species has
405 previously been identified as a *C. gigas* pathogen [48], and its increase in relative abundance
406 in our sequencing data is consistent with the higher *Vibrio* bacterial counts in disease-affected
407 samples from this specific site [17]. Furthermore, a prior study has demonstrated that the
408 hemolymph microbiomes of *Vibrio*-infected *C. gigas* showed an increase in bacteria from

409 potentially pathogenic genera, such as *Photobacterium,* and bacteria belonging to *Bacteroidia*,
410 *Clostridia*, *Propionigenium*, *Vibrio*, *Arcobacter* and *Mollicutes* [27]. It is notable that similar
411 increases in bacteria belonging to these groups were observed in this study, with an unidentified
412 member of the *Mollicutes* identified as being a core member of disease-affected microbiomes.
413 While it is not possible to determine to what extent these *Vibrio* OTUs caused this oyster
414 mortality event, our observations of their elevated abundance in diseased oysters and evidence
415 from previous work [7, 27, 31] points towards a potential role in infection or opportunistic
416 colonisation.
417
418 Sewage associated bacteria were found to be significantly elevated in disease-affected samples
419 at Site A, in particular the *Paludibacter* genus 31616 was found to be completely absent in the
420 unaffected samples, while those assigned to the *Bacteroides* genus 94495 were significantly
421 elevated in disease-affected samples at both Site A and in the southern region as a whole. The
422 *Paludibacter* genus has been associated with animal waste [49, 50], while the *Bacteroides*
423 genus is found to be heavily abundant in the human gastrointestinal tract (GIT) (reviewed by
424 [51]) and can be used as an indicator for human faecal contamination [49, 52]. This over-
425 representation of sewage/faecal associated OTUs in the southern site may be explained by the
426 geographical features of the Port Stephens estuary, with Tilligerry creek having a strong
427 agricultural and mining industrial presence on the creek, as well as being exposed to effluent
428 off-flow from septic systems during periods of high rainfall [34]. Interestingly, there was a
429 small rainfall event of 12.6mm approximately eight days before the start of the first mortalities
430 at Cromartys Bay. Tilligerry creek flows out towards the northeast [34], as Cromartys Bay sits
431 at the mouth of Tilligerry creek, it is possible that water entering Cromartys Bay influenced
432 the bacterial communities in that bay as well. Due to their elevation in only the disease-affected

samples, it may be possible that unaffected oysters had greater capability to flush out these bacteria from their tissues.

Conclusion

During a major summer mortality event that occurred among the commercial *C. gigas* stocks, we observed substantial variability in the oyster microbiome between individuals, sites and disease states. These variations were characterised by changes in the relative abundance of abundant bacterial groups including those members from the *Brachyspiraceae* family, *Mycoplasma* genus, *Mollicutes* class, *Bacteroidales* order and the *Paludibacter* genus. In addition, rare OTUs belonging to *V. harveyi* and an unidentified member of the *Vibrio* genus were found to be significantly more abundant in disease-affected oyster microbiomes at Site A. Due to the acute and sporadic nature of mortality events, samples could only be collected as the outbreak was occurring. Future studies aimed at conducting a temporal study to observe the stability of the microbiome before an outbreak and to determine whether shifts occur before or during the disease outbreak will provide further insights into the role of shifting oyster microbiome structure in summer mortality events.

Acknowledgments

We would like to acknowledge the New South Wales Department of Primary Industries for their assistance in collecting and processing the oyster samples, as well as their continued support over the length of the project. This research was supported by an Australian Research Council Linkage Project LP160101785 and partly funded by Ausgem, a research partnership initiated between the University of Technology Sydney and the New South Wales Department of Primary Industries.

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Supplementary Table 1. Comparisons of alpha and beta diversity between disease-states and locations.

Comparison	p-value
Disease-unaffected Vs. South disease-affected	0.0002 (SIM)
Disease-unaffected Vs. North disease-affected	0.0001 (SIM)
North disease-affected Vs. South disease-affected	0.0001 (SIM)
Species diversity North disease-affected Vs. South disease-affected	0.047 (ANO)
Species richness North disease-affected Vs. South disease-affected	0.00046 (ANO)
Species evenness North disease-affected Vs. South disease-affected	0.31 (ANO)
Hatchery disease-affected Vs. Wild-sourced disease-affected	0.0052 (SIM)
Species diversity SA disease-affected Vs. SA disease-affected	0.46 (ANO)
Species richness SA disease-affected Vs. SA disease-affected	0.019 (ANO)
Species evenness SA disease-affected Vs. SA disease-affected	0.84 (ANO)
SA disease-affected Vs. SA disease-affected	0.0073 (SIM)
SA disease-affected Vs. SA disease-affected similarity index	0.005 (KS)
SF disease-affected Vs. SA disease-affected similarity index	0.007 (KS)
Northern Vs. Southern similarity index	0.29 (KS)
North disease-affected Vs. South disease-affected similarity index	0.009 (KS)

KS represents a Kruskal-Wallis ANOVA

SIM represents a one-way ANOSIM

ANO represents a one-way ANOVA

SA represents Cromartys bay Site A

SF represents Tilligerry creek Site F

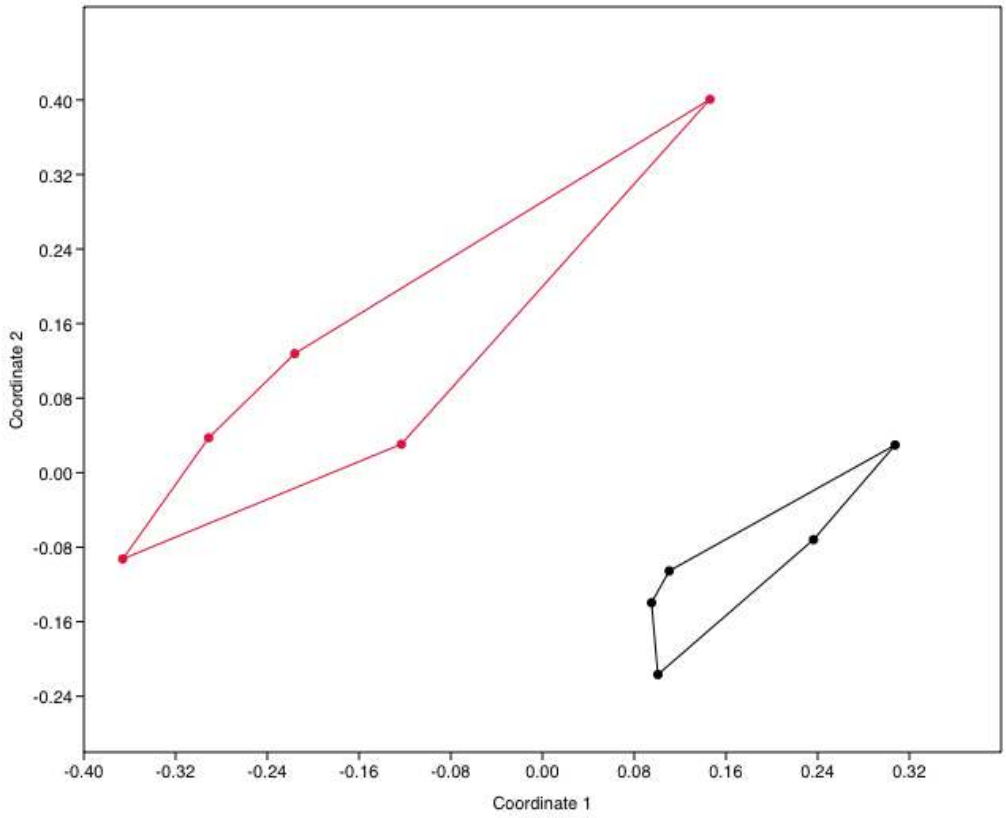
Supplementary Table. 2 Core microbiome of disease-affected and disease-unaffected oyster microbiomes. Unique OTUs are those OTUs exclusively found in that disease state, while shared OTUs are those found in both disease states. Analysis was run using the panbiom.py script [1], with a core OTU member defined as an OTU present in 80% of samples.

Disease-state	Unique OTUs		
Disease-affected	*Mollicutes* 89399		
Disease-unaffected	*Phaeobacter* 20291		*Rubrobacter* 66142
	Brachyspiraceae 24319		*Erythrobacteraceae* 70953
	Pseudoalteromonas 38778		*Arcobacter* 85107
	Pseudoalteromonas 65827		*Vibrio fortis* 109919
Shared	*Mycoplasma* 3538	*Brachyspiraceae* 32677	*Synechococcus* 73807
	Octadecabacter 8040	*Mycoplasma* 38764	*Vibrio shilonii* 78468
	Pseudoalteromonadaceae 18290	*Bradyrhizobium* 47243	*Mycoplasma* 85564
	Pseudoalteromonas 18539	*Jannaschia* 52393	*Erythrobacter* 96008
	Polynucleobacter 20780	*Polaribacter* 54209	*Helicobacter* 99035

Supplementary Table. 3 SIMPER analysis of disease-affected oyster microbiomes (northern or southern region) compared to disease-unaffected oyster microbiomes. The top 10 OTUs are displayed with their dissimilarity contribution and mean transformed representation.

OTU	Dissimilarity (%)	North disease-affected mean	Disease-unaffected mean
Brachyspiraceae 32677	2.70	4.93	3.6
Mycoplasma 38764	2.65	2.42	1.7
Spirochaetes 20129	1.76	0.45	0.90
Mycoplasma 3538	1.51	1.41	0.85
Pseudoalteromonadaceae 18290	1.37	0.47	1.46
Mollicutes 89399	1.37	1.18	0.47
Mycoplasma 78519	1.25	1.11	0.76
Mycoplasma 85564	1.23	0.85	0.83
Polynucleobacter 20780	1.21	1.07	0.93
Alphaproteobacteria 556	1.02	0.78	0.38
OTU	Dissimilarity (%)	South disease-affected mean	Disease-unaffected mean
Brachyspiraceae 32677	2.50	1.68	3.6
Mycoplasma 38764	2.08	1.28	1.7
Spirochaetes 20129	2.03	1.01	0.90
Pseudoalteromonadaceae 18290	1.67	1.76	1.46
Bacteroidales 50223	1.35	1.42	0.14
Mycoplasma 85564	1.25	1.16	0.83
Bacteroidales 27418	1.03	1.09	0.04

Bacteroides 94495	1.01	1.01	0.05
Mycoplasma 3538	1.00	0.59	0.85
Alphaproteobacteria 556	0.96	0.50	0.38

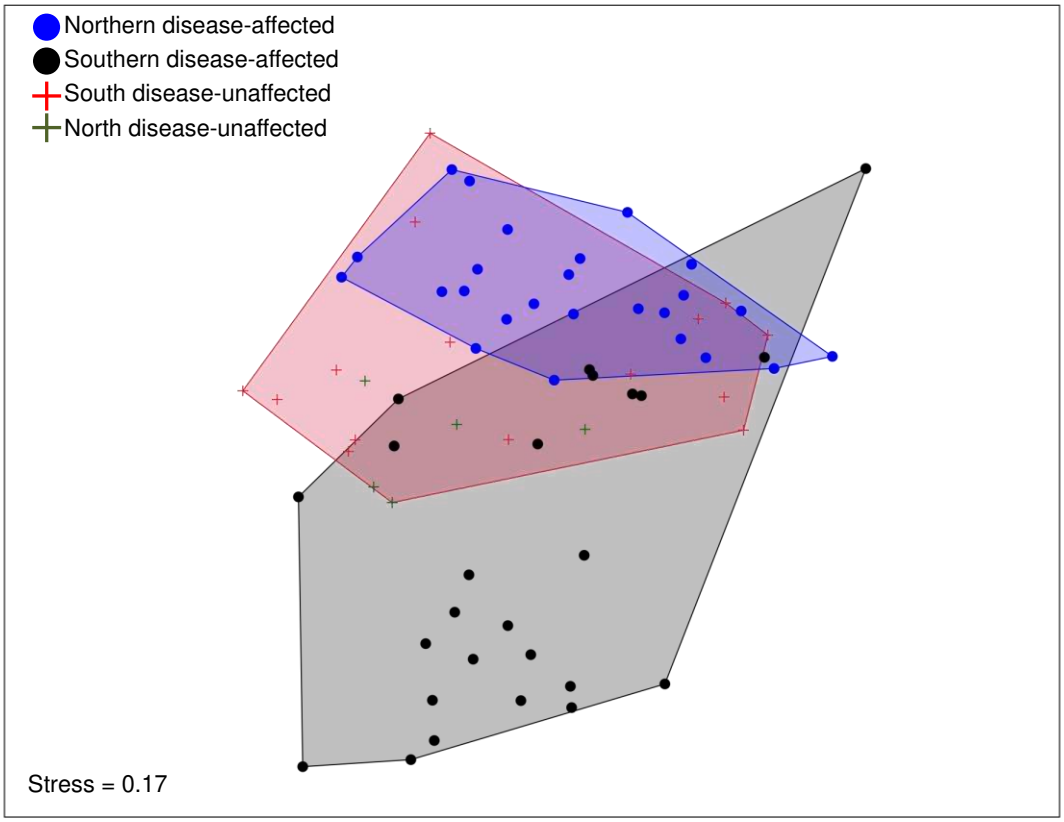


Supplementary Fig. 1. nMDS plot showing disease state separation between *C. gigas* microbiomes at Cromartys Bay Site A. Disease-affected samples are represented as black circles and are outlined in black. Unaffected samples are represented as red crosses and are outlined in red. Samples separate based on disease state. Stress 0.084

Supplementary Table. 4 SIMPER analysis of Cromartys bay Site A. The top 10 OTUs are displayed with their dissimilarity contribution and mean transformed representation in either disease-affected or disease-unaffected samples

OTU	Dissimilarity contribution (%)	Disease-affected mean	Disease-unaffected mean
Pseudoalteromonadaceae 18290^	2.556	3.09	0.766
Spirochaetes 20129	1.938	0.0928	1.43
Bacteroidales 27418^	1.916	2.16	0.147
Brachyspiraceae 32677	1.791	1.78	2.75
JTB215 100999*^	1.74	1.74	0
Bacteroidales 86667^	1.738	1.71	0
Paludibacter 31616^	1.667	1.77	0
Mycoplasma 38764	1.398	0.0635	1.3
Bacilli 86663	1.345	0	1.02
Bacteroides 94495^	1.262	1.39	0.132

*Of the order *Clostridiales*
^Significantly more abundant with a Kruskal-Wallis ANOVA ($p < 0.05$)



Supplementary Fig. 2. nMDS plot showing spatial and disease state separation. Samples separate spatially based on region, as well as by their disease state. Axes 1 and 3 are plotted.

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1. Kahlke T (2017) Panbiom. https://githubcom/timkahlke/panbiom.