Regional and oyster microenvironmental scale heterogeneity in the Pacific oyster bacterial community

William L King[1, 2†], Nachshon Siboni[2], Tim Kahlke[2], Michael Dove[4], Wayne O'Connor[4], Khandaker Rayhan Mahbub[1], Cheryl Jenkins[3], Justin R Seymour[2], Maurizio Labbate[1*]

[1]University of Technology Sydney, The School of Life Sciences, Ultimo NSW 2007, Australia

[2]University of Technology Sydney, Climate Change Cluster, Ultimo NSW 2007, Australia

[3]NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales, Australia

[4]NSW Department of Primary Industries, Port Stephens Fisheries Institute, Port Stephens, New South Wales, Australia

[†]Current address: Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA, USA

* Correspondence:

Postal address: University of Technology Sydney, PO Box 123, Broadway NSW, 2007, Australia

Telephone number: +61 2 9514 4064 14

Email: maurizio.labbate@uts.edu.au

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Abstract

Different organs of a host represent distinct microenvironments resulting in the establishment of multiple discrete bacterial communities within a host. These discrete bacterial communities can also vary according to geographical location. For the Pacific oyster, *Crassostrea gigas*, the factors governing bacterial diversity and abundance of different oyster microenvironments are poorly understood. In this study, the factors shaping bacterial abundance, diversity and composition associated with the *C. gigas* mantle, gill, adductor muscle, and digestive gland were characterised using 16S (V3-V4) rRNA amplicon sequencing across six discrete estuaries. Both location and tissue-type, with tissue-type being the stronger determinant, were factors driving bacterial community composition. Bacterial communities from wave-dominated estuaries had similar compositions and higher bacterial abundance despite being geographically distant from one another, possibly indicating that functional estuarine morphology characteristics are a factor shaping the oyster bacterial community. Despite the bacterial community heterogeneity, examinations of the core bacterial community identified *Spirochaetaceae* bacteria as conserved across all sites and samples. Whereas members of the *Vulcaniibacterium*, *Spirochaetaceae* and *Margulisbacteria*, and *Polynucleobacter* were regionally conserved members of the digestive gland, gill, and mantle bacterial communities respectively. This indicates that baseline bacterial community profiles for specific locations are necessary when investigating bacterial communities in oyster health.

Introduction

In many marine plants and animals, the bacterial community is an important determinant of host health and physiology (Rosenberg *et al.*, 2007, Tarnecki *et al.*, 2017, Crump *et al.*, 2018, Pita *et al.*, 2018), with shifts in composition increasingly being linked to disease (De Lorgeril *et al.*, 2018, King *et al.*, 2019). These shifts in bacterial community composition are observed in a number of marine benthic organisms including tubeworms (Vijayan *et al.*, 2019), corals (Woo *et al.*, 2017, Marcelino *et al.*, 2018) and seagrasses (Cúcio *et al.*, 2016), and are driven by broad-scale external processes such as seasonal changes (Sharp *et al.*, 2017), geographic location (Cúcio *et al.*, 2016, Woo *et al.*, 2017) and time (Vijayan *et al.*, 2019). Despite the apparent inherent variability within bacterial communities, phylotypes are often conserved over large geographic scales (Ainsworth *et al.*, 2015) and time periods (Aronson *et al.*, 2017).

A host organism's bacterial community can also vary within an individual (Ainsworth *et al.*, 2015, Marcelino *et al.*, 2018) due to distinct microenvironments represented by different host tissues or organs (Ainsworth *et al.*, 2015, Crump *et al.*, 2018, Marcelino *et al.*, 2018). These host-associated (individual-scale) microenvironments may accommodate a range of ecological interactions between the host organism and its microbial consortia (Jensen *et al.*, 2007, Ainsworth *et al.*, 2015, Brodersen *et al.*, 2018, Marcelino *et al.*, 2018), important for key physiological processes such as nitrogen fixation in seagrasses (Lehnen *et al.*, 2016), nutrient uptake in corals (Ainsworth *et al.*, 2015) and seagrasses (Harlin, 1973), and host defences in tunicates and sponges (Florez *et al.*, 2015). Therefore, broad-scale and individual-scale processes act in concert to shape the host bacterial community. Henceforth, we will refer to seasons and locations as broad-scale influences and different tissues (or organs) within an organism as individual-scale influences.

Among marine benthic organisms, there is growing evidence for the importance of the bacterial community in *Crassostrea gigas* (the Pacific Oyster) health, particularly within the context of disease dynamics (Trabal *et al.*, 2012, Wegner *et al.*, 2013, Lemire *et al.*, 2015, Lokmer & Wegner, 2015, Petton *et al.*, 2015, Lokmer *et al.*, 2016a, De Lorgeril *et al.*, 2018, King *et al.*, 2019). *C. gigas* is a significant contributor to aquaculture production and economic output representing one of the most heavily cultivated species globally. However, recurrent disease outbreaks in recent decades have compromised output (Burge *et al.*, 2006, Soletchnik *et al.*, 2007, Malham *et al.*, 2009, Mortensen *et al.*, 2016, Go *et al.*, 2017). The factors governing the structure of the *C. gigas* bacterial community are poorly understood but emerging evidence suggests that the *C. gigas* bacterial community is dynamic and influenced by location (Lokmer *et al.*, 2016a, Lokmer *et al.*, 2016b), time (Lokmer *et al.*, 2016a, Lokmer *et al.*, 2016b) and genetics (Wegner *et al.*, 2013). Additionally, within a given location at a specific time, the *C. gigas* bacterial community displays substantial heterogeneity among individuals (Lokmer *et al.*, 2016b, King *et al.*, 2019) and between different oyster tissues within individuals (Lokmer *et al.*, 2016b).

Understanding the factors governing the structure of the *C. gigas* bacterial community is important because there is evidence suggesting that the bacterial community is a factor in oyster disease dynamics (Wegner *et al.*, 2013, Lokmer & Wegner, 2015, De Lorgeril *et al.*, 2018, Green *et al.*, 2019, King *et al.*, 2019). While these studies have provided evidence that the oyster bacterial community may be intimately involved in polymicrobial infection dynamics (Petton *et al.*, 2015, De Lorgeril *et al.*, 2018), there are limited culture-independent studies examining the bacterial community of *C. gigas* without the confounding influence of disease (Lokmer *et al.*, 2016a, Lokmer *et al.*, 2016b). Identifying a 'healthy' or 'normal' baseline bacterial community and defining the dynamics (e.g. physiological and

environmental factors) that influence its structure is essential when aiming to interpret its role in health including susceptibility to pathogens.

We examined the bacterial community of four different oyster tissues across six different estuaries spanning four degrees of latitude along the eastern coastline of Australia (New South Wales, Australia). Our principal goals were to understand: (i) the spatial influence on bacterial community composition, (ii) the influence of oyster microenvironments (tissue types) on bacterial community assemblage and (iii) conservation of the oyster bacterial community across oyster microenvironments and locations. This information provides the foundation for disentangling the role of the bacterial community in both oyster health and disease.

Methods

Oyster collection sites and sampling

To examine the spatial heterogeneity of oyster bacterial communities, *C. gigas* samples were collected from six oyster farms along the east coast of New South Wales (NSW), Australia (Figure 1), spanning a distance of approximately 470 kilometres. Starting from the southernmost location, the sampled environments included: The Wapengo lagoon, Clyde River, Shoalhaven River (Crookhaven river), Georges River, Hawkesbury River, and Port Stephens. The Clyde River is the largest producer of *C. gigas* representing 41 % of all oysters produced in NSW, followed by Port Stephens (27 %), the Hawkesbury River (9 %), and the Shoalhaven River (9 %) (DPI, 2019). All sampling locations are tide-dominated drowned valley estuaries (Roy *et al.*, 2001), except for the Wapengo and Shoalhaven sites, which are wave-dominated barrier estuaries (Roy *et al.*, 2001). Both estuary-types are characterised by high river flows but differ with the levels of marine flushing, with wave-dominated estuaries having less marine flushing relative to tide-dominated estuaries.

Ten adult oysters were collected from each of these sites during a six-day period in August 2018. Samples were immediately frozen (-20 °C), transported to the laboratory in a portable freezer and stored at -20 °C prior to analysis.

Extraction of DNA from different oyster tissues

We examined the bacterial community associated with four different oyster tissue types, including the mantle, gill, adductor muscle, and digestive gland (inclusive of digestive diverticula). Ten oyster samples from each location were rinsed under running tap water to remove any external debris and mud. Thawed oysters were then shucked using sterile shucking knives and placed in sterile petri dishes. Oysters were weighed and approximately 25 mg of each respective tissue was dissected and removed from each oyster sample with sterile scalpel blades. DNA was then extracted from the 240 individual tissue samples using the Qiagen DNeasy Blood and Tissue Kit (catalogue: 69506), as per the manufacturer's instructions.

Quantitative PCR (qPCR)

To provide an indication of the bacterial abundance within each sample, we employed a quantitative PCR (qPCR) assay to quantify total 16S rRNA gene copies. An epMotion 5075l Automated Liquid Handling System integrated with a Bio-Rad CFX384 Touch Real-Time PCR Detection System was used to perform the analysis. All analyses were performed with three technical replicates with a standard curve and negative controls, using the following reaction mixture: 2.5 μL iTaq Universal SYBR Green supermix, 0.2 μL of each 10 μM forward and 10 μM reverse primer, 1 μL of template DNA, and 1.1 μL of sterile water. Bacterial abundance was quantified using the 16S rRNA specific primers BACT1369F (CGGTGAATACGTTCYCGG) and PROK1492R (GGWTACCTTGTTACGACTT) (Suzuki *et al.*, 2000). The qPCR cycling conditions were: 95 °C for 3 minutes followed by 45 cycles

of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds, generating a 142 bp product. The resulting data were normalised to tissue weight. A coefficient of variation (CV) was then calculated for the technical triplicates, and where necessary, samples with CV > 2 % had a replicate removed from the analysis. A melting curve was added to the end of every run to confirm the presence of a single PCR product.

Oyster bacterial community analysis

The microbial community composition of each oyster tissue was characterised with 16S rRNA amplicon sequencing, using the 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primer pair (Herlemann *et al.*, 2011) targeting the V3-V4 region of the 16S rRNA gene. The PCR cycling conditions generating the 16S rRNA amplicons were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Sequencing was performed using the Illumina MiSeq platform 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) under Bioproject number PRJNA551083.

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME 2 version 2018.6.0) pipeline (Bolyen *et al.*, 2018). Briefly, paired-ended 16S DNA sequences were imported using the 'qiime tools import' command. Sequences were then trimmed and denoised using DADA2 version 1.6, which also removes chimeras (Callahan *et al.*, 2016). Taxonomy was then assigned at the single nucleotide level using the classify-consensus-vsearch qiime feature classifier against the Silva v132 database (Quast *et al.*, 2013). Sequences identified at the single nucleotide threshold are henceforth denoted as ZOTUs (zero-radius OTUs; Edgar (2016)). For those ZOTUs with poor taxonomic assignment, the representative sequence was blasted against the NCBI database. The dataset

was further cleaned by removing ZOTUs with less than 400 reads and those identified as chloroplasts or mitochondria. Cleaned data were then rarefied at 8,100 reads per sample corresponding to a threshold that permitted the inclusion of 5 or more replicate samples for every tissue type (217 remaining samples; Supplementary Table 1).

Core bacterial community analysis

To determine whether a core bacterial community was conserved for a given tissue type across all sampling environments, we used the panbiom.py analysis described in Kahlke (2017). The analysis was performed with the following parameters: abundance minimum of 0.0 (-m parameter) and a replicate threshold corresponding to 80% (-r parameter)). A core ZOTU was defined as a ZOTU present in 80 % of the tested samples to account for outliers.

Statistical analysis

Alpha diversity measures, including species diversity (Shannon's index), species evenness, and species richness (observed species) were calculated and compared in the QIIME 2 statistical environment. Alpha diversity measures were compared between locations (15 tests) or between tissue-types (6 tests) using a Kruskal-Wallis test. Further, alpha diversity data were analysed with a linear mixed model generated in R using the lme function within the nlme package. Model fit was statistically tested using the anova.lme function within the nlme package. To compare community structure between sampling locations and tissue types, normalised data (square root (x)) were first compared using non-metric multidimensional scaling analysis (nMDS) with a Bray-Curtis similarity index. Microbial assemblages were subsequently compared using a one-way PERMANOVA with a Bray-Curtis similarity index to elucidate significant bacterial community patterns across tissue types and sampling locations. To identify the variance explained (R^2) between statistical comparisons, adonis within the vegan package was used with 999 permutations. To identify which bacterial taxa

were driving the difference between locations and tissue types, a similarity percentage analysis (SIMPER) with a Bray-Curtis similarity index was used with data summarised at the genus level or at the ZOTU level. Comparisons of 16S rRNA gene copies (16S rRNA qPCR) were first performed with a Kruskal-Wallis statistical test followed by a Mann-Whitney pairwise test. All beta diversity (nMDS, PERMANOVA, and SIMPER) and qPCR comparisons were performed in the PAST statistical environment (Hammer *et al.*, 2001). To account for multiple testing, uncorrected p-values were adjusted using the false discovery rate (Benjamini-Hochberg) to generate q-values.

Results

Patterns in Bacterial abundance inferred from 16S rRNA qPCR

Estimates of bacterial abundance, as determined by qPCR of the 16S rRNA gene, were highest in oysters collected from the Shoalhaven and Wapengo sampling locations (Figure 2; Supplementary Table 2) and differed significantly between the other sampling locations (Kruskal-Wallis ANOVA; $H = 87$; $p < 0.0001$). Overall, the mantle tissue had the highest number of 16S rRNA gene copies compared to all other tissues ($H = 40$; $p < 0.0001$; Supplementary Table 3) with the mantle and gill tissues at each sampling location generally having higher 16S rRNA gene copies compared to the adductor muscle and digestive gland (Supplementary Table 4). The mantle and gill samples from Wapengo and Shoalhaven both had consistently higher numbers of 16S rRNA gene copies compared to all other tissues at all other sites except for the adductor muscle tissue at Shoalhaven (Supplementary File 2).

Alpha diversity comparisons

Species richness, evenness, and diversity were all significantly different between sampling locations (richness $H = 98$, $p = < 0.0001$; evenness $H = 32$, $p = < 0.0001$; diversity $H = 70$, p

= < 0.0001). Oyster bacterial communities from the Wapengo and Shoalhaven locations displayed the highest levels of species richness and diversity (Supplementary Table 5; Supplementary Figure 1; Supplementary File 2). Oyster bacterial communities from the Wapengo, Shoalhaven, and Hawkesbury River locations had the highest species evenness. In agreement, a linear mixed model identified Wapengo and Shoalhaven as having the highest species diversity and species richness, respectively (Supplementary File 2). In addition, the Hawkesbury river had the highest species evenness, closely followed by Wapengo and Shoalhaven (Supplementary File 2).

All measured alpha diversity indices were also significantly different between tissue-types (richness H = 24, p = < 0.0001; evenness H = 17, p = 0.0008; diversity H = 28, p = < 0.0001). The digestive gland bacterial community displayed the highest levels of species richness (Supplementary Table 6). Species evenness was similar across the gill, adductor muscle, and digestive gland, but was significantly lower in the mantle. Similarly, the mantle tissue had the lowest levels of species diversity, with highest diversity levels within the digestive gland and gill (Supplementary Table 6). In agreement, linear mixed models identified the digestive gland tissue as having the highest species diversity and species richness (Supplementary File 2). Further, no difference was observed with species evenness between the gill, adductor muscle and digestive gland tissues, but it was significantly lower in the mantle tissue (Supplementary File 2).

Geographic location and tissue type are significant determinants of the C. gigas bacterial community

The structure of the *C. gigas* bacterial community differed significantly according to both sampling location (one-way PERMANOVA; F = 11; p = 0.0001; R^2 = 0.14) and the tissue type (F = 13.6; p = 0.0001; R^2 = 0.11) (Table 1 and 2). However, despite these statistical

differences, clear partitioning of the microbial assemblages was not evident when all data was included in an nMDS analysis (stress 0.28; Supplementary File 2), highlighting the highly heterogenous nature of the oyster bacterial community.

To further resolve the influence of tissue type or sampling environment on the oyster bacterial community structure we compared the bacterial communities of different tissue types within, and between, sampling environments. In the first instance, tissue-specific oyster bacterial communities differed significantly from each other within all locations (Figure 3; Clyde River $F = 4.5$, $p = 0.0001$, $R^2 = 0.18$; Georges River $F = 5$, $p = 0.0001$, $R^2 = 0.15$; Hawkesbury River $F = 3.6$, $p = 0.0001$, $R^2 = 0.18$; Port Stephens $F = 5.2$, $p = 0.0001$, $R^2 = 0.19$; Shoalhaven $F = 3.9$, $p = 0.0001$, $R^2 = 0.18$; Wapengo $F = 3.9$, $p = 0.0001$, $R^2 = 0.19$). Notably, significant differences were identified between all pairwise comparisons between tissues within all sites (Supplementary File 2) implying a strong tissue-type influence on the oyster bacterial community.

When using data summarised at the genus level, uncultured *Spirochaetaceae* bacteria were the strongest driver of tissue-specific bacterial community differences within sites (Figure 4), contributing 10.7 % to the dissimilarity between tissues (Table 3), primarily due to their over-representation in the mantle tissue. Members of the *Mycoplasma* and *Vulcaniibacterium* genera were responsible for 6.1 % and 4.8 % of the dissimilarity contribution between tissues, primarily due to an overabundance of these genera in the digestive gland. Members of the *Spirochaetaceae* family and the *Margulisbacteria* phylum were responsible for 2.4 %, and 2.1 % of the bacterial community variability between tissues respectively, predominantly due to their over-representation in the gill tissue. Members of the *Acidovorax* genus accounted for 4.5 % of the bacterial community dissimilarity between tissues and were most abundant in adductor muscle and digestive gland bacterial communities. The *Polynucleobacter* genus accounted for 3.4 % of the dissimilarity contribution between tissue-

types and were most abundant in the mantle. At the ZOTU level, the top eight contributors were ZOTUs assigned as uncultured *Spirochaetaceae* bacteria, which cumulatively contributed 11.24 % to the bacterial community dissimilarity and were all over-represented in the mantle tissue (Supplementary File 2).

Tissue-specific bacterial communities differed significantly between sampling locations (Figure 3; Mantle $F = 5.3$, $p = 0.0001$, $R^2 = 0.17$; Gill $F = 4$, $p = 0.0001$, $R^2 = 0.14$; Adductor muscle $F = 5.3$, $p = 0.0001$, $R^2 = 0.21$; Digestive gland $F = 4.4$, $p = 0.0001$, $R^2 = 0.20$), further confirming the regional-scale spatial variability of the *C. gigas* bacterial community composition. However, when examining pairwise comparisons of specific tissues between individual locations, the mantle, gill, and digestive gland bacterial communities from Wapengo were not significantly different to the same tissue types at the Shoalhaven site (Supplementary File 2). These similarities are perhaps notable, given that the Wapengo and Shoalhaven sites are the only two sampled sites characterised as wave-dominated estuaries. Pairwise comparisons of adductor muscle bacterial communities from the tide-dominated estuaries were not significantly different between the Georges River site when compared to the Clyde River and Port Stephens sites. Further, the adductor muscle bacterial community from the Hawkesbury River was not significantly different to those from Port Stephens. As expected, these data suggest a regional influence on the oyster bacterial community composition, though it is notable that these large-scale differences in bacterial community structure were not as strong as the microenvironmental-scale, tissue-type influence.

Uncultured *Spirochaetaceae* bacteria contributed to the greatest dissimilarity between oyster bacterial communities from different sampling locations, accounting for 10 % of the variability between sites, largely due to a relative over-abundance of these bacteria in the Clyde River, Georges River, Hawkesbury River, and Port Stephens (Table 4). Bacteria assigned to the *Vulcaniibacterium* genus were responsible for 5.2 % of the bacterial

community variability between sites, driven by an over-representation in the Wapengo and Shoalhaven sampling location bacterial communities. At these sites, members of the *Limnobacter* and *Pseudoxanthomonas* genera were also over-represented, contributing 4.5 % and 4.1 % to the bacterial community dissimilarities, while they were completely absent, or in low abundance, at the other four sampling locations. Bacteria assigned to the *Vibrio* genus were over-represented in the adductor muscle and digestive gland bacterial communities at the Clyde River site, relative to all other locations, contributing 1.1 % of the dissimilarity between bacterial communities. Members of the SAR11 clade contributed 1 % to the dissimilarity between sites and were common across the Clyde River, Georges River, Hawkesbury River, and Port Stephens sites but were almost completely absent in the Wapengo and Shoalhaven sites. When examining ZOTU level SIMPER comparisons, the top seven contributors were ZOTUs assigned as uncultured *Spirochaetaceae* bacteria, which cumulatively contributed 9.8 % to the bacterial community dissimilarity and were all over-represented in the tide-dominated estuaries (Supplementary File 2).

Conservation of the C. gigas core bacterial community

As the structure of the oyster bacterial community was governed by both the sampling location and tissue type, we sought to identify core bacterial communities for (i) all of the tested oyster bacterial communities regardless of tissue type or location (universal core bacterial community), (ii) each sampling location regardless of tissue type, and (iii) each tissue type regardless of location (Figure 5). When including all samples in the core analysis, several ZOTUs assigned to an uncultured *Spirochaetaceae* (ZOTUs 04655, 29fe1, fe651, 3bb6f, 295f6, 80d03, 1986e, and a9435) were characterised as members of the 'universal' core bacterial community, whereby they were found in at least 80% of all tested samples, regardless of sampling location or tissue type.

The oyster bacterial communities from the Wapengo and Shoalhaven sampling locations harboured a distinct core bacterial community relative to the other sampling sites. This (Wapengo-Shoalhaven) core bacterial community was consistent across all tissue types and included ZOTUs assigned to the *Acidovorax* (ZOTUs f83c7 and e7d4f), *Vulcaniibacterium* (ZOTUs eaa6d and 6b014), *Pseudoxanthomonas* (ZOTU 39c33), *Limnobacter* (ZOTUs 35f52 and 0d183), and *Sphingomonas* (ZOTU 03a2c) genera.

Individual tissues were also found to harbour unique core bacteria. In addition to the *Spirochaetaceae* ZOTUs identified in the universal core bacterial community, the mantle and gill tissues consisted of other uncultured *Spirochaetaceae* bacteria (ZOTUs ecd55 and 922bd; ZOTUs b51b6 and a4b53 respectively). No additional core ZOTUs were identified in the adductor muscle bacterial community. No core bacterial community was identified for the digestive gland, however, slightly relaxing the core analysis parameters from 80% (present in 40/50 samples) to 78% (present in 39/50 samples) allowed for the inclusion of ZOTUs classified as members of the *Vulcaniibacterium* (ZOTUs eaa6d and 6b014) and *Delftia* (ZOTUs b37a8 and 75c38) within the core bacterial community of the digestive gland.

Discussion

Our principal aim was to characterise the *C. gigas* bacterial community from four different oyster tissues in oysters collected from six different estuaries to understand the influence of space and tissue microenvironments on the oyster bacterial community assemblage. Location and oyster tissue type were significant determinants of the oyster microbial assemblage, although similarities in bacterial community structure were observed between geographically distant locations with similar estuary characteristics. Despite the observed oyster bacterial community heterogeneity, conserved members, such as ZOTUs assigned to the *Spirochaetaceae* family, were also identified.

Both location and tissue type influence the oyster bacterial community assemblage

Geographic location has previously been found to influence the haemolymph, mantle, gill (Lokmer *et al.*, 2016a, Lokmer *et al.*, 2016b) and disease-affected adductor muscle bacterial communities (King *et al.*, 2019). Consistent with these studies, we observed a significant effect of location on the oyster bacterial community. However, bacterial community similarities between the mantle, gill, and digestive gland bacterial communities from Shoalhaven and Wapengo locations (wave-dominated estuaries), and between the adductor muscle bacterial communities at the remainder of the sampling locations (tide-dominated estuaries) over large geographic distances, suggests that geographic location is only one factor driving heterogeneity in the bacterial community. These data suggest that estuary-type influences the bacterial community composition and should be considered when examining patterns in bacterial community heterogeneity between individuals. The oyster bacterial community assemblage was also influenced by the oyster tissue, with each tissue harbouring a unique microbial consortia, as previously observed (Lokmer *et al.*, 2016b). This pattern was observed for all pairwise comparisons within all locations, suggesting that tissue-type is a stronger driver of bacterial community composition than geographic location.

Estuary properties and their potential influence on the oyster bacterial community

Similarities between the bacterial communities from the Wapengo and Shoalhaven sites were surprising, given the distance between sampling sites (approximately 200 km). These two sites shared a core bacterial community not observed in any other sampling locations, and displayed no significant bacterial community differences between the mantle, gill, and digestive gland bacterial communities. With members of the *Vulcaniibacterium*, *Limnobacter* and *Pseudoxanthomonas* genera representing the predominate taxa driving the differences between both Wapengo and Shoalhaven, and the other four sampling locations. The

Shoalhaven site has a catchment size of 7, 500 km^2 (Roy *et al.*, 2001), with approximately 35 % of the catchment used for agricultural purposes (OceanWatch-Australia, 2017). In contrast, Wapengo has a significantly smaller catchment of 73 km^2 (Roy *et al.*, 2001) but a similar level of agricultural usage at 20 % (OceanWatch-Australia, 2010). Both sites have a high proportion of forest/undisturbed area with approximately 50 % of the catchment at the Shoalhaven site and 70 % at the Wapengo site (OceanWatch-Australia, 2010, OceanWatch-Australia, 2017). As both sampling locations are shallow wave-dominated estuaries (Roy *et al.*, 2001), it is possible that the reduced marine flushing and high river flow introduces more soil-associated microbes into the water column from the river, which settle and allow the oysters to consume them. This could explain the higher relative abundance of soil associated microbes (i.e. *Vulcaniibacterium* and *Pseudoxanthomonas* bacteria) (Yoo *et al.*, 2007, Young *et al.*, 2007, Wei *et al.*, 2012, Yu *et al.*, 2013) and a higher general abundance of microbes (16S rRNA gene counts) at these sites compared to the other tide-dominated locations and may explain the similarities in bacterial community composition between them. The efficient marine flushing of tide-dominated estuaries could also explain the higher abundance of the SAR11 clade in these sites. Future studies should aim to characterise the involvement of marine flushing and river flow on the oyster bacterial community, and whether carry-over from taxa in the soil have implications for oyster health.

Of the sampled locations, the Clyde River represents the most 'pristine' environment (Rubio *et al.*, 2008). The Clyde River catchment spans an area of 1, 791 km^2 (Roy *et al.*, 2001), of which, 95 % comprises of forest/undisturbed area and 4 % for agricultural/rural usage (Cavanagh *et al.*, 2004). Previous studies comparing the Shoalhaven and Clyde River identified that oysters grown in the Shoalhaven grew approximately 27 % faster than the counterparts in the Clyde River (Rubio *et al.*, 2008). Increased growth rates in the Shoalhaven were attributed to increased nutrient loads and on average, higher water

temperature (Rubio *et al.*, 2008). Bacterial communities from the Clyde River were dominated by uncultured *Spirochaetaceae* bacteria, and the adductor muscle and digestive gland bacterial communities at this site were markedly over-represented by *Vibrio* bacteria when compared to all other locations. The reduced growth rate and lower nutrient loads could act as a stressor for oysters at the Clyde River site, possibly allowing *Vibrios* to colonise and proliferate (Lemire *et al.*, 2015, Bruto *et al.*, 2017, De Lorgeril *et al.*, 2018, King *et al.*, 2019).

Oyster tissue bacterial community heterogeneity

Given the conservation of bacterial communities associated with specific tissues across geographically discrete locations, it is likely that the type of oyster tissue is a stronger driver of bacterial community composition than geographic location. Several ZOTUs were most responsible for driving the differences between tissue-types and may be important in tissue-specific processes. Of these, ZOTUs classified as members of the *Mycoplasma* and *Vulcaniibacterium* genera were over-represented in the digestive gland. *Mycoplasma* are commonly identified in the oyster digestive system (Green & Barnes, 2010, King *et al.*, 2012), but the *Vulcaniibacterium* genus is a newly described group and only includes two species (Yu *et al.*, 2013). Members of the *Spirochaetaceae* family and the *Margulisbacteria* phylum were over-represented in the gill. While we observed a strong connection between spirochaete taxa and the gill bacterial community, there are conflicting reports with previous studies often observing these taxa in the oyster digestive gland (Green & Barnes, 2010), oyster homogenates (Fernandez-Piquer *et al.*, 2012) or the adductor muscle (King *et al.*, 2019, King *et al.*, 2019). This is likely due to the high taxonomic classification of the *Spirochaetaceae* family, as it could represent a diverse range of different oyster-associated microbes. Further, little is known about the *Margulisbacteria* phylum however, a previous study observed physical attachment of a *Margulisbacteria* bacteria to an ectosymbiotic

spirochaete bacteria in termite guts (Utami *et al.*, 2019) possibly explaining their co-dominance with bacteria assigned to the *Spirochaetaceae* family in the oyster gill bacterial community. Bacteria assigned to the *Polynucleobacter* genus and an uncultured *Spirochaetaceae* were over-represented in the mantle. *Polynucleobacter* species have previously been observed in oyster homogenate bacterial communities (Fernandez-Piquer *et al.*, 2012), this genus contains both obligate endosymbionts of ciliates (Heckmann & Schmidt, 1987, Vannini *et al.*, 2005) and planktonic bacteria (Hahn *et al.*, 2010). Finally, members of the *Acidovorax* genus were over-represented in the adductor muscle and digestive gland bacterial communities. Members of the *Acidovorax* have been isolated from a diverse range of environments including soil (Chaudhary & Kim, 2018), water (Pal *et al.*, 2018), and from cyanobacterial blooms (Chun *et al.*, 2017).

Conservation of Spirochaete ZOTUs across sampling environments and tissue types

Despite the significant heterogeneity in the oyster bacterial community across environments and tissue types, we did identify core taxa associated with all locations and tissue types. Several ZOTUs, classified as *Spirochaetaceae* bacteria were consistent members of the *C. gigas* core bacterial community across all sites and tissues. Blasting the representative sequences for these ZOTUs, identified these uncultured spirochaete bacteria previously in *C. gigas* in Tasmania, Australia (Fernandez-Piquer *et al.*, 2012), as well as in *C. gigas* in Germany and the Netherlands (Lokmer *et al.*, 2016a), and in *Saccostrea glomerata* in Queensland, Australia (Green & Barnes, 2010), indicating a very wide geographical distribution of these core oyster associates. Furthermore, we previously identified these bacteria as members of the core bacterial community in Port Stephens oyster bacterial communities (OTUs 32677 and 24319 (King *et al.*, 2019)), although these organisms were assigned as members of the *Brachyspiraceae* family. This discrepancy is likely attributed to previously using the Greengenes database for taxonomy assignment, as opposed to the

SILVA database in this study. We also previously found it associated with OsHV-1 disease-resistant oysters (OTU 4737 (King *et al.*, 2019)). Apart from its presence in different oyster bacterial community datasets across different countries and locations within Australia, little is known about these bacteria. Future studies should attempt to further phylogenetically characterise these bacteria and identify their potential functional role(s) within *C. gigas*.

Conclusions

Emerging evidence suggests that the oyster bacterial community is dynamic, shaped by a range of broad- and individual-scale processes however, elements such as estuarine morphology and hydrodynamics have yet to be considered as influencing the bacterial community. Our analysis revealed that the structure of the *C. gigas* microbial assemblage is governed by both geographic location and tissue type, with bacterial communities derived from wave-dominated estuaries exhibiting similar bacterial community assemblages despite large geographic separation, with a predominance of soil/particulate-associated bacteria within these bacterial communities. Given the dynamic nature of oyster bacterial communities, our understanding of whether the oyster bacterial community has conserved elements across regions or microenvironments is lacking. We revealed a core bacterial community within individual tissue-types, and a universal core bacterial community consisting of uncultured *Spirochaetaceae*, as conserved across all sampling locations and tissue types. This finding was strengthened by the presence of this taxa in other previously published oyster bacterial community datasets. Due to the dynamic nature of the bacterial community, and the strong effect of location and tissue-type on the oyster bacterial community, it is difficult to interpret disease-affected bacterial communities based on oyster bacterial communities from different locations or tissues. Instead, future studies should aim to characterise the healthy bacterial communities of oysters for the specific location where oysters are grown to use as a reference during disease events. Further, the characterisation of

temporal bacterial community patterns with sufficient sampling resolution during baseline 'healthy' periods can be used to examine seasonal bacterial community variability before disease periods.

Data availability

All raw sequencing data in FASTQ format were deposited in the NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA551083.

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Author contributions

WK and NS processed the samples. WO'C and MD collected the samples. WK, NS and KM analysed the data. CJ, MD, WO'C, JS, and ML designed the study. TK produced the core bacterial community analysis. WK, JS, and ML wrote the manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any competing interests, including commercial or financial relationships that could be construed as a potential conflict of interest.

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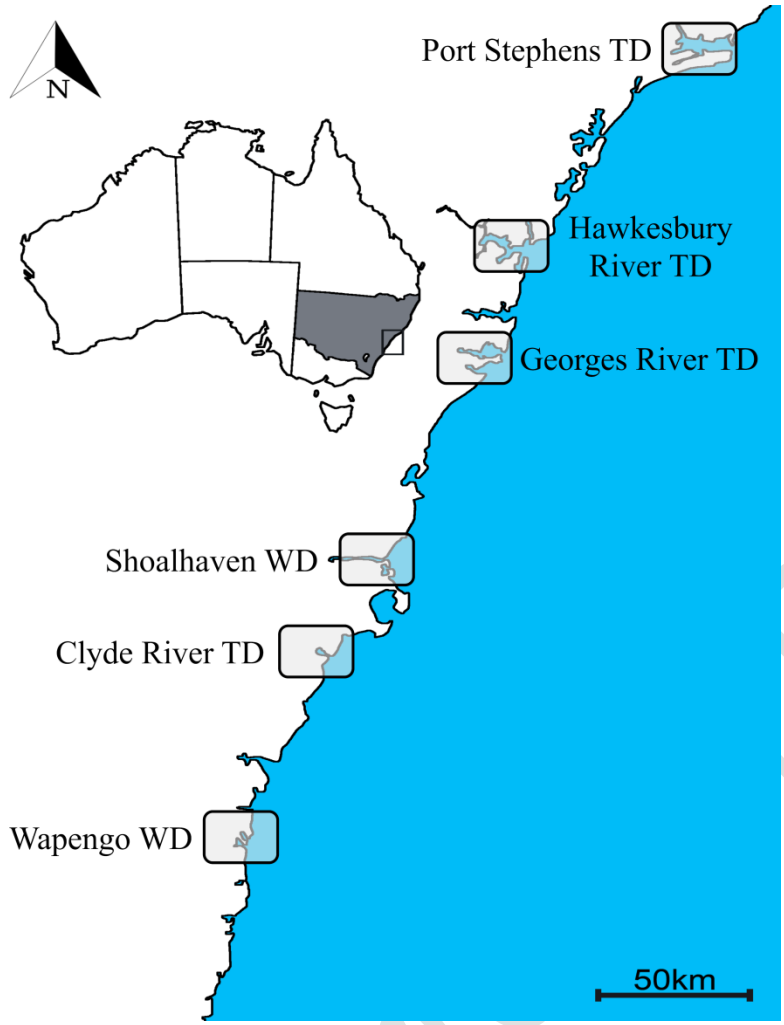

Figure 1: Sampling locations across New South Wales, Australia. Ten adult oysters from each location were sampled for bacterial community characterisation (see Supplementary File 2 for final sample numbers). TD and WD represents a tide-dominated and wave-dominated estuary, respectively.

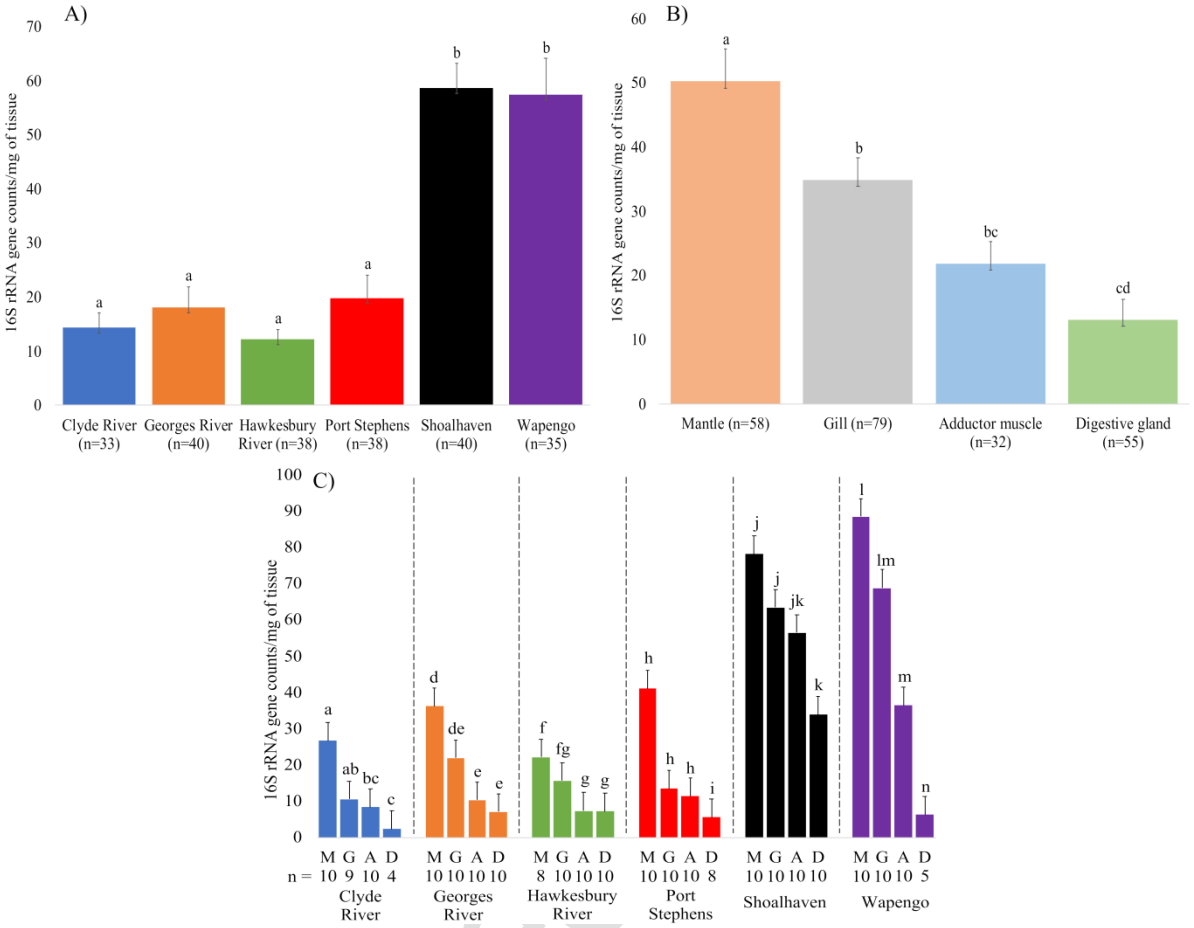

Figure 2: 16S rRNA copy number of oysters and their tissues. Data are average 16S rRNA counts per milligram of tissue with standard error. A) 16S rRNA counts per location. B) 16S rRNA counts per tissue type. C) 16S rRNA counts for each tissue at each location. Significant comparisons are denoted by different letters. Displayed comparisons for section C) were performed within locations.

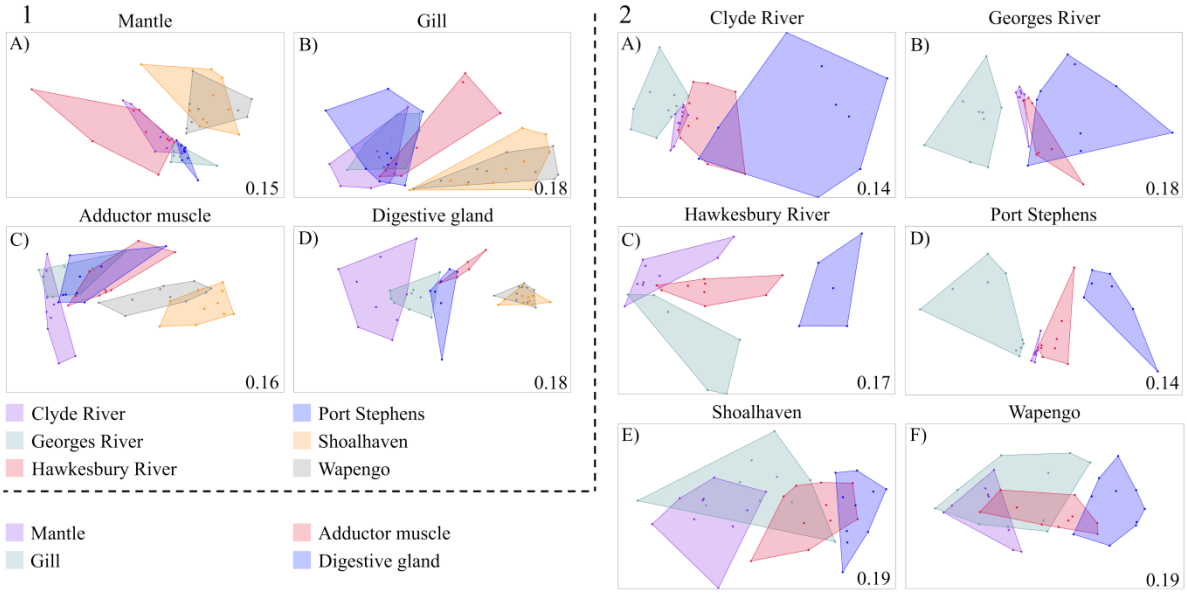

Figure 3: nMDS plots of oyster tissue-type bacterial communities at individual locations (panel 1) and at different locations (panel 2). For panel 1: Clyde River = purple, Hawkesbury River = red, Georges River = green, Port Stephens = blue, Shoalhaven = orange, and Wapengo = grey. For panel 2: Mantle tissues = purple, gill tissues = green, adductor muscle tissues = red, and digestive gland tissues = blue. Stress values are provided in the lower right corner of each subfigure.

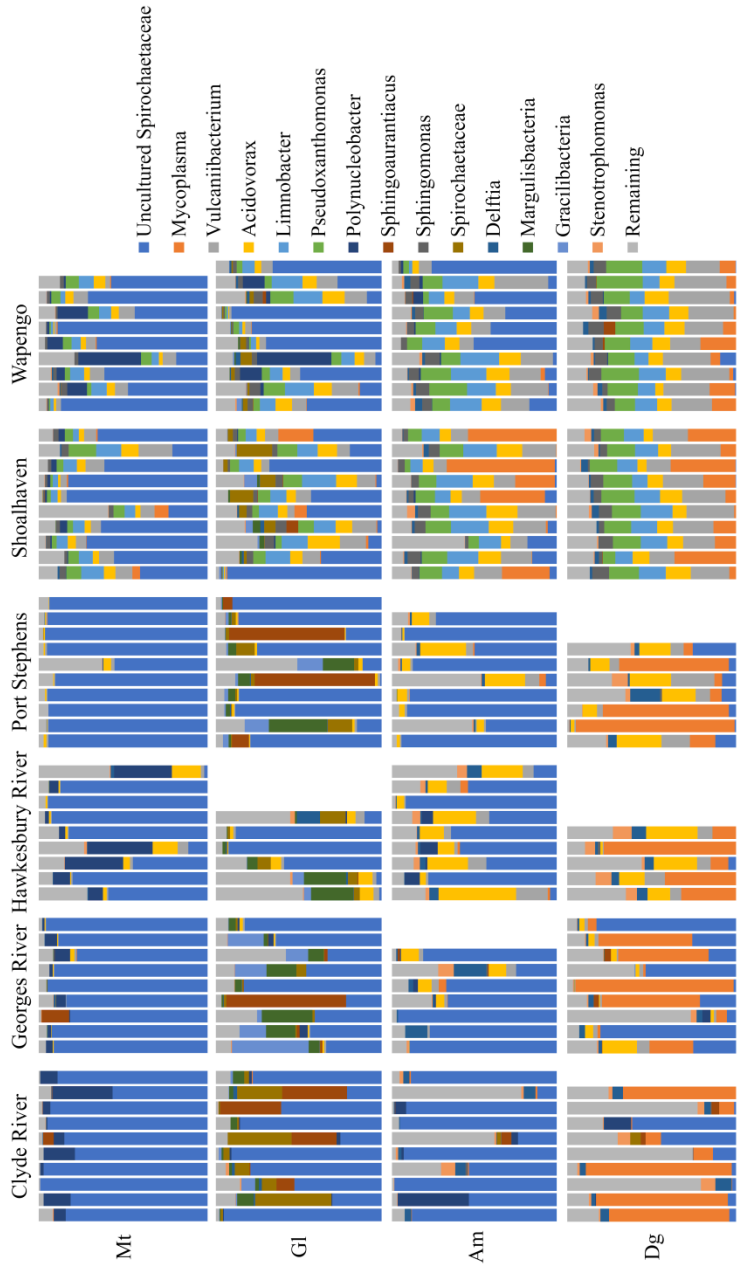

Figure 4: Summarised oyster bacterial communities at the genus level, across six sampling locations and four sampled tissues. Tissues are labelled as: Mt = mantle, Gl = gill, Am = adductor muscle and Dg = digestive gland. Top 15 summarised genera are shown with the remaining genera grouped together (other).

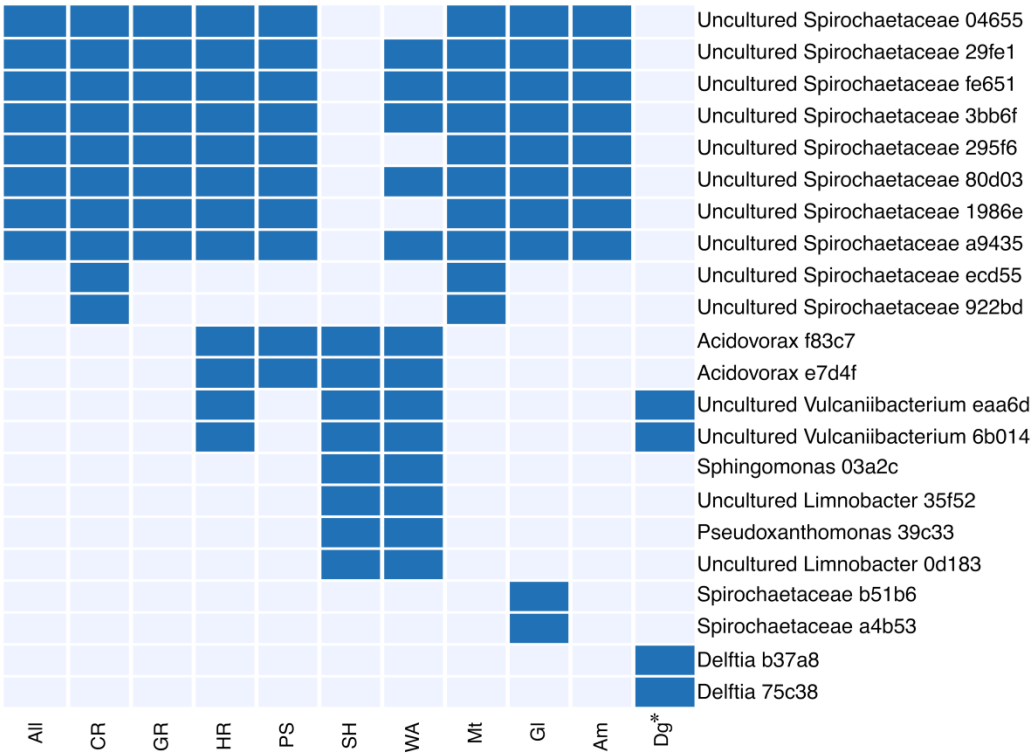

Figure 5: Presence/absence heatmap of taxa identified as the core bacterial community. Dark blue boxes represent the presence of a core taxa. Core bacterial community analyses were performed using i) all samples regardless of location or tissue, ii) individual sites regardless of tissue and iii) individual tissues regardless of location. All = All samples were included in the analysis, CR = Clyde River, GR = Georges River, HR = Hawkesbury River, PS = Port Stephens, SH = Shoalhaven, and WA = Wapengo. Mt = mantle, Gl = gill, Am = adductor muscle, Dg = digestive gland. *Criteria were slightly relaxed to 78 % for the digestive gland core.

Table 1: One-way PERMANOVA pairwise comparisons between sampling locations, including the q-value, F-value and R^2 value (adonis)

Location	Clyde river	Georges river	Hawkesbury river	Port Stephens	Shoalhaven
Georges river	0.0003; 3.9; 0.06				
Hawkesbury river	0.0008; 6.2; 0.1	0.0003; 4.7; 0.08			
Port Stephens	0.0005; 5.3; 0.1	0.028; 2; 0.05	0.001; 3.6; 0.06		
Shoalhaven	0.0004; 23.9; 0.26	0.0002; 19.9; 0.24	0.0001; 13.1; 0.18	0.0002; 18.3; 0.2	
Wapengo	0.0003; 18.9; 0.23	0.0002; 15.7; 0.21	0.002; 10.9; 0.16	0.0002; 14.8; 0.18	0.006; 2.6; 0.04

Table 2: One-way PERMANOVA pairwise comparisons between tissue types, including the q-value, F-value and R^2 value (adonis)

Tissue type	Mantle	Gill	Adductor muscle
Gill	0.0006; 12.7; 0.11		
Adductor muscle	0.0003; 8.7; 0.09	0.0002; 9.5; 0.09	
Digestive gland	0.0002; 27; 0.26	0.0001; 15.8; 0.15	0.0001; 8.4; 0.08

Table 3: SIMPER analysis between grouped tissue types using data summarised to the genus level, including the taxa, dissimilarity contribution, and the mean abundance for each tissue-type (%).

Taxa	Dissimilarity contribution (%)	Mantle mean	Gill mean	Adductor muscle mean	Digestive gland mean
Uncultured *Spirochaetaceae* family	10.71	74	48.8	47.4	10.5
Mycoplasma genus	6.068	0.30	0.63	4.33	26.6
Vulcaniibacterium genus	4.797	2.87	3.43	7.59	12.2
Acidovorax genus	4.466	2.39	3.5	8.91	8.61
Limnobacter genus	3.889	2.17	3.74	5.64	5.19
Pseudoxanthomonas genus	3.651	1.67	2.19	4.22	6.68
Polynucleobacter genus	3.36	6.51	1.71	1.87	0.45
Spirochaetaceae family	2.426	0.025	5.63	0.053	0.13
Delftia genus	2.426	0.19	0.70	2.17	2.83
Sphingomonas genus	2.407	0.70	1.18	1.9	2.41
Margulisbacteria	2.124	0.020	5.03	0.003	0

phylum					

Table 4: SIMPER analysis between grouped sampling locations using data summarised to the genus level, including the taxa, dissimilarity contribution, and the mean abundance (%) for that location. CR is Clyde river, GR is the Georges river, HR is the Hawkesbury river, SH is the Shoalhaven site, PS is the Port Stephens site, and WA is the Wapengo site.

Taxa	Dissimilarity contribution (%)	CR mean	GR mean	HR mean	PS mean	SH mean	WA mean
Uncultured *Spirochaetaceae* family	9.966	59.1	60.4	43.4	59.6	24.5	33
Mycoplasma genus	5.371	8.64	9.03	6.36	7.54	10.7	2.34
Vulcaniibacterium genus	5.164	0	1.26	4.38	2.89	13.7	14.3
Acidovorax genus	4.71	0	2.51	11.3	6.06	8.28	7.39
Limnobac	4.481	0	0	0	0.014	12.1	10.6

ter genus							
Pseudoxanthomonas genus	4.133	0	0	0	0.001	9.78	10
Polynucleobacter genus	3.347	4.6	1.48	5.74	0	0.47	4.5
Sphingomonas genus	2.714	0.053	0.032	0.056	0.045	4.03	4.15
Delftia genus	2.268	1.63	1.67	2.39	1.07	0.95	1.14
Vibrio genus	1.231	4.03	0.074	0.10	0.073	0.075	0.14
SAR11 clade	1.014	0.19	0.208	0.47	0.56	0.015	0.006