Dynamics of the Sydney rock oyster microbiota before and during a QX disease event

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Abstract

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is the most intensively farmed oyster species in Australia however, Queensland unknown (QX) disease has resulted in substantial losses and impeded productivity. QX disease is caused by infection with the parasite *Marteilia sydneyi,* and like other diseases, outbreaks are driven by a series of complex environmental and host factors such as seasonality, seawater salinity and oyster genetics. A potential but understudied factor in QX disease is the SRO microbiota, which we sought to examine before and during a QX disease outbreak. Using 16S rRNA (V1 – V3 region) amplicon sequencing, we examined the microbiota of SROs deployed in an estuary where QX disease occurs, with sampling conducted fortnightly over 22 weeks. *Marteilia sydneyi* was detected in the SROs by PCR (QX-positive) 16 weeks after the first sampling event and sporonts were observed in the digestive gland two weeks later on. There were no apparent patterns observed between the microbiota of QX-positive SROs with and without digestive gland sporonts however, the microbiota of QX-positive SROs was significantly different from those sampled prior to detection of *M. sydneyi* and from those negative for *M. sydneyi* post detection. Therefore, shifts in microbiota structure occurred before sporulation in the digestive gland and either before or shortly after pathogen colonisation. The microbiota shifts associated with QX-positive oysters were principally driven by a relative abundance increase of operational taxonomic units

(OTUs) assigned to unclassified species of the *Borrelia* and *Candidatus Hepatoplasma* genera and a relative abundance decrease in an OTU assigned to an unclassified species of the *Mycoplasma* genus. Since *Mycoplasma* species are common microbiota features of SROs and other oysters, we propose that there may be an important ecological link between *Mycoplasma* species and the health state of SROs.

Keywords: *Saccostrea glomerata,* QX disease, *Marteilia sydneyi,* selective breeding

1. Introduction

The Sydney Rock Oyster (SRO; *Saccostrea glomerata*) is native to Australia, where it is the most intensively cultivated oyster species (O'Connor & Dove, 2009; Schrobback *et al.*, 2014). However, the SRO industry has been significantly impacted by a disease called QX (Queensland Unknown Disease), which has caused annual losses of SRO stocks of up to 100% in some cultivation regions (Peters & Raftos, 2003). QX disease was first detected in the late 1960s in Moreton Bay in the north-eastern Australian state of Queensland (Wolf, 1972). Since the late 1970s, QX has extensively spread across Queensland (Adlard & Ernst, 1995) and southwards into several New South Wales estuaries (Nell, 2007; Raftos *et al.*, 2014). The disease is caused by a spore-forming protozoan parasite called *Marteilia sydneyi* that initiates its infection in the oyster's palps and gills as a uninucleate stem cell, and then over several weeks, migrates through connective tissue and the haemolymph into the digestive gland (Wolf, 1979; Kleeman *et al.*, 2002). Once in the digestive gland, the parasite undergoes sporulation, forming mature sporonts containing two tricellular spores (Wolf, 1979; Kleeman *et al.*, 2002) and causing blockage in the digestive gland resulting in starvation and death (Wolf, 1979).

Notably, the presence of *M. sydneyi* within an SRO farming estuary does not necessarily result in a QX disease outbreak (Adlard & Wesche, 2005) indicating that other factors, beyond the presence of the pathogen, are important for infection or progression of disease. For example, infection is thought to require an intermediate host(s) (Raftos *et al.*, 2014). As with other oyster diseases, QX disease is likely driven by a convergence of environmental (e.g. water chemistry, temperature), host-specific (e.g. immunity and stress level) and pathogen-specific factors (Green *et al.*, 2011; Raftos *et al.*, 2014; King *et al.*, 2019b). QX disease is seasonally recurrent, generally occurring in summer or autumn (depending on the location) (Wolf, 1979; Adlard &

Ernst, 1995; Nell, 2007; Rubio *et al.*, 2013). Additionally, low seawater salinity is considered a major contributing factor (Lester, 1986; Rubio *et al.*, 2013) possibly through its inhibition of phenoloxidase (PO) activity in SROs (Butt *et al.*, 2006), an enzyme in invertebrates that initiates host immune defences (Söderhäll & Cerenius, 1998). Decreased PO activity in SROs is known to be associated with increased susceptibility to QX disease (Peters & Raftos, 2003; Butt & Raftos, 2007) although the full mechanism(s) by which PO is involved in QX disease resistance is unresolved.

Within the more studied Pacific oyster (*Magallana gigas*, formally *Crassostrea gigas*) system, the microbiota is emerging as a key factor in disease dynamics (Petton *et al.*, 2015; King *et al.*, 2019b). For example, Pacific oysters with common genetics but varying microbiota have different mortality outcomes when challenged with the viral pathogen OsHV-1 (Pathirana *et al.*, 2019). This is possibly explained by the fact that OsHV-1 supresses Pacific oyster immunity allowing opportunistic pathogens such as *Vibrio* species to infect (de Lorgeril *et al.*, 2018). If Pacific oysters contain lower levels of opportunistic pathogens in their microbiota then they are less likely to be exposed to bacterial infection post OsHV-1 infection (Petton *et al.*, 2015; King *et al.*, 2019c; Pathirana *et al.*, 2019). Additionally, other studies have made links between the oyster microbiota and disease (Lokmer & Wegner, 2015; King *et al.*, 2019d), including one study that demonstrated the progressive replacement of a benign *Vibrio* population in the Pacific oyster microbiota with a virulent population during a mortality outbreak (Lemire *et al.*, 2015) and suggesting that non-virulent bacteria may facilitate the disease of virulent bacteria (Lemire *et al.*, 2015). Given the importance of the oyster microbiota within the disease dynamics of other oyster species, we propose that shifts in the SRO microbiota might also play a role in QX disease.

Previously, a clone library-based approach demonstrated that that the digestive gland microbiota of SROs containing sporulating *M. sydneyi* is significantly different from uninfected oysters, with QX infected oysters dominated by an OTU closely related to a member of the *Rickettsiales* (Green & Barnes, 2010). As sporulation in the digestive gland occurs in the late stages of QX disease, it is not possible to know if this OTU emerged prior to infection or as a consequence of infection and, whether it has a role in facilitating infection or driving QX disease progression. Using 16S rRNA amplicon sequencing, we have recently shown that the SRO microbiota associated with the adductor muscle is dominated by OTUs assigned to

unclassified species of the *Candidatus Hepatoplasma*, *Endozoicomonas* and *Mycoplasma* genera, and that the microbiota is significantly influenced by location and season (Nguyen *et al.*, 2020). Additionally, we found that selective breeding of SROs for QX disease resistance influences the structure of the microbiota, but only in winter before the typical QX disease period (late summer or early autumn) with OTUs assigned to unclassified species of the *Mycoplasma*, *Borrelia* and *Endozoicomonas* genera over-represented in the QX resistant SRO microbiota and OTUs assigned to unclassified species of the *Pseudoalteromonas*, *Vibrio*, and *Candidatus Hepatoplasma* genera over-represented in QX sensitive SRO microbiota (Nguyen *et al.*, 2020). During this previous work, the SROs were deployed in non-QX disease areas and only two time points (one time point each in the Austral summer and winter) were compared therefore, a more comprehensive investigation of the SRO microbiota in QX disease dynamics is warranted. Here we employed fortnightly sampling to examine temporal shifts in the SRO microbiota before and during a QX disease event.

2. Materials and methods

2.1. Experimental design and sampling

A QX disease field challenge was performed in the Georges River, New South Wales (NSW), Australia (33°59'19"S 151°03'21"E), which is a high risk site for QX disease and has been used to develop QX disease resistance in SROs since 1997 (Nell & Perkins, 2006; Dove et al., 2013; Dove *et al.*, 2020). Four SRO families (F32, F43, F48 and F67) sourced from the NSW Department of Primary Industries SRO Breeding Program were used. The four families were from the 2016-year class and were predicted to have intermediate levels of survival (20-50%) over the course of a QX disease outbreak and were selected to allow comparisons of infected and uninfected SRO microbiota. Three replicate groups for each family were deployed using the standard method for a QX disease exposure trial to measure survival through a QX disease outbreak (Dove *et al.*, 2020). Additional oysters from each family were deployed to collect periodic samples for analyses.

Oyster families were deployed on the 20th of September 2017 and left to acclimatise for 7 weeks, well before the expected QX disease period at this site which generally occurs in February (Nell & Perkins, 2006; Dove *et al.*, 2013). Sampling was initiated on the 8th of November 2017. Since sporonts of the *M. sydneyi* parasite are identifiable in the digestive gland

approximately 2 weeks after initial QX detection (Peters & Raftos, 2003) and the infection lasts weeks to months (Rubio *et al.*, 2013), fortnightly sampling was determined to be of sufficient resolution for capturing and following a QX mortality event. Initially, five oysters per family per sampling time were collected, but to permit comparison of SRO microbiota with and without sporonts in the digestive gland, this was increased to ten oysters once the QX pathogen was detected by PCR (see below). Oysters were randomly collected from cultivation trays, placed into a labelled plastic bag, kept on ice, and immediately transported to the laboratory (within two hours). Before the QX disease event, oysters were stored at -80 ^{0}C and then thawed for tissue excision and DNA extraction. Once the QX pathogen was detected, fresh oysters were immediately processed to check the digestive gland for sporonts via tissue imprinting (see below). Other tissues were excised and frozen in cryotubes at -80 ^{0}C for later DNA extraction. For each sampling time, 10 L of seawater was collected at a depth of 10 to 20 cm, kept on ice and transported to the laboratory. Triplicate aliquots of 200 mL seawater were filtered with glass microfiber filters (0.7 µm pore size) for subsequent chlorophyll-a analysis and, triplicate aliquots of 2 L seawater were filtered with Durapore Membrane Filters (0.22 µm pore size) for DNA extraction. These filters were frozen in liquid nitrogen and kept at -80 ^{0} C prior to further processing. For nutrient analysis, triplicate 50 mL water samples were syringe filtered through a 0.45 µm filter into 50 mL sterile falcon tubes in the field, transported to the laboratory on ice, and frozen at -20^0 C.

2.2. Measurement of environmental parameters, nutrients and chlorophyll a in water

Environmental parameters (temperature, oxygen, pH, and conductivity) were measured at the time of collection using a WTW multiprobe meter (Multi 3430, Germany) at time of collection. Nutrient analyses (nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) and phosphate (PO_4^{3-})) were conducted at Envirolab Services Pty Ltd (Sydney, NSW, Australia). Chlorophyll a was analysed based on a spectrophotometric method described previously (Ritchie, 2006).

2.3. DNA extractions and 16S rRNA amplicon sequencing

Oysters (frozen oysters were thawed first) were washed under running tap water to remove debris. Using sterile instruments, each oyster was carefully opened using a shucking knife and

the oyster flesh excised and placed onto a sterile Petri dish. Approximately 25-50 mg of adductor muscle tissue was then excised using a sterile scalpel blade and placed into a tube. Additional tissues consisting of 10 – 15 mg of adductor muscle, gill and digestive gland were excised and pooled into a separate tube. All samples underwent DNA extraction using the Qiagen DNeasy Blood and Tissue DNA extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA from filtered seawater samples was extracted using the PowerWater DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocol.

Extracted adductor muscle DNA samples were subjected to PCR targeting the ribosomal 16S rRNA V1–V3 region using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′) primer pair (Lane, 1991; Turner *et al.*, 1999) attached to Illumina MiSeq barcodes. PCR and sequencing were performed using the Illumina MiSeq v3 2×300 bp platform at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) following the manufacturer's guidelines. Raw data files in FASTQ format were deposited in the NCBI Sequence Read Archive with the study accession number SRP266167 under Bioproject number PRJNA637460.

2.4. Detection of *Marteilia sydneyi* in oyster tissue and sporont production in the digestive gland

Oysters were confirmed as infected with *M. sydneyi* by PCR using the primers LEG1 (5'-CGATCTGTGTAGTCGGATTCCGA) and PRO2 (5'-TCAAGGGACATCCAACGGTC) (Kleeman & Adlard, 2000) using the pooled adductor muscle, gill and digestive gland DNA extract as a template. Each PCR reaction contained 1 µL DNA (25 – 50 ng), 10 µL MangoMix (Bioline), 1 µL LEG1 primer (10 µM stock), 1 µL PRO2 primer (10 µM stock) and 7 µl water to a total of 20 µL. The PCR cycling conditions were as follows: 94 ^{0}C for 2 min, followed by 30 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec and 72 ^{0}C for 30 sec with a final extension of 72 ^{0}C for 10 min. DNA extracted from an oyster confirmed to be infected with *M. sydneyi* was used as a positive control. PCR products were electrophoresed on a 1 % (w/v) agarose gel alongside a low molecular weight DNA marker. During the QX disease event, all SROs were tested for the presence of sporulating *M. sydneyi* in the digestive gland by using the tissue imprint method described in (Kleeman & Adlard, 2000) using the Rapid Diff kit (Australia Biostain company) for staining.

205
206 **2.5. Bioinformatics and statistical analyses**
207
208 To increase reads per sample, oyster DNA samples were sequenced twice, resulting in four
209 FASTQ files per sample. Complementary reads were concatenated to create paired-end reads
210 per sample. Demultiplexed paired-end reads were combined using FLASH (Magoč & Salzberg,
211 2011) and filtered by length and quality scores (Parameters: maxhomop = 5, maxambig = 0,
212 minlength = 471, maxlength = 500) using Mothur (Schloss *et al.*, 2009). Fragments were
213 clustered into OTUs at 97 % sequence similarity, and chimeric and singletons sequences were
214 identified and removed using VSEARCH (Rognes *et al.*, 2016). Taxonomic assignment of
215 OTUs were performed in QIMME version 1.9.1 (Caporaso *et al.*, 2010) using the UCLUST
216 algorithm (Edgar, 2010) against the SILVA v128 dataset (Quast *et al.*, 2013). Mitochondrial
217 and chloroplast data were filtered out of the dataset. Alpha diversity indices, including Chao1,
218 Simpson and Shannon were calculated using QIIME (Caporaso *et al.*, 2010).
219
220 For alpha diversity, a Kruskal-Wallis test was used to identify differences in species richness
221 (Chao1), species evenness (Simpson) and species diversity (Shannon). For beta diversity,
222 samples that had less than 1,000 reads were removed, remaining data normalized using the
223 proportion method (McKnight *et al.*, 2019) and then OTUs with less than 0.1% relative
224 abundance were filtered out. All analyses were performed with a Bray-Curtis dissimilarity
225 index. Non-metric multidimensional scaling (nMDS) analysis was used to elucidate patterns
226 between sample groups. To determine if microbiota were significantly different, a One-way
227 PERMANOVA with 9999 permutations was used with normalised (square root (x)) data. A
228 Similarity Percentages (SIMPER) test was used to identify the observed dissimilarity of the
229 bacterial communities between groups. These statistical analyses were performed using the
230 PAST version 3.24 statistical environment (Hammer *et al.*, 2001). To determine whether OTUs
231 were significantly different between oyster groups, a Welch's t-test was performed using
232 STAMP software package version 2.1.3 (Parks *et al.*, 2014). To identify significant
233 associations between environmental variables, *M. sydneyi* and the SRO microbiota, a
234 correlation analysis was performed using the MICtools software package with default
235 parameters (Albanese *et al.*, 2018). Significant correlations with a SpearmanRho ≥ 0.1 and $\leq$
236 - 0.1 were kept for further analyses (Akoglu, 2018). All OTUs with less than 1% in relative
237 abundance were filtered out prior to analysis and explanatory variables for inclusion in the
238 analysis (i.e. QX infection) were binary transformed. Network models were used to visualise

significant correlations by using Cytoscape software version 3.6.1 (Su *et al.*, 2014) was used to visualise significant network correlations.

3. Results

3.1. Sample categorisation and, sequence reads and data filtering

SROs were deployed on the 20th of September 2017 with fortnightly sampling commencing on the 8th of November 2017. The QX disease parasite was detected in oysters by PCR approximately 16 weeks later on the 27th of February 2018 signalling the start of a QX disease event. Prior to QX detection, a total of 160 oysters had been collected over 16 weeks and were categorised as "Pre-QX". During this QX disease event, a total of 140 oysters were collected at four discrete sampling times, of which 77 were classified as negative and 63 positive for *M. sydneyi* by PCR (Supplementary Figure 1A) and categorised as "QX-negative" and "QX-positive" respectively. Of the QX-positive SROs, 24 were positive for the presence of mature sporonts in the digestive gland as determined by tissue imprint (Supplementary Figure 1B). These SROs were categorised as "QX-sporonts" and defined as being infected with numbers consistent with the average survival (± SD) of the families following the QX outbreak measured in July 2018 of 83 ± 3 % for F32, 64 ± 10 % for F64, 81 ± 15 % for F48 and 81 ± 18 % for F67. The average survival of the 62 families in the 2016-year class was 81 % which indicates that this QX disease outbreak was less severe compared to previous seasons (Dove *et al.*, 2020).

Following amplicon sequencing of the 336 samples and removal of samples with less than 1,000 reads, a total of 2,306,494 reads were obtained from 298 samples (262 SROs and 36 seawater samples; Supplementary Table 1). After data filtering, a total of 3,750 OTUs were identified across the entire dataset with 3,492 and 87 OTUs unique to the oyster and seawater microbiota respectively and, 171 OTUs common to both. Of the 262 oysters, 148 oysters were Pre-QX with the remaining 114 oysters collected after detection of QX disease, with these consisting of 52 QX-positive and 62 QX-negative (Supplementary Table 1). Of the 52 QX-positive SROs, 28 were negative and 24 were positive for mature sporonts in the digestive gland (QX-sporonts).

3.2. The SRO and seawater microbiota are distinct

Across the dataset, species richness (Chao1), evenness (Simpson) and diversity (Shannon) were significantly higher in the seawater samples compared to the SRO microbiota (Supplementary Figure 2 and Supplementary Table 2, $p < 0.001$ for all comparisons). An nMDS plot grouped the SRO and seawater microbiota separately (Supplementary Figure 3) with PERMANOVA confirming that the microbiotas were significantly different ($F = 53.58$, $p < 0.001$). SIMPER analysis identified a 98.9 % dissimilarity between the seawater and SRO microbiota. OTUs assigned to unclassified species of the *Mycoplasma* (OTU 11355) and *Candidatus Hepatoplasma* (OTU 11357) genera were over-represented in SROs contributing 9.26 % and 6.65 % of the dissimilarity respectively. OTUs assigned to unclassified species of the *Candidatus Actinomarina* (OTU 16613) and NS5 marine group (OTU 4487) genera were over-represented in seawater contributing 7.14 % and 4.64 % of the dissimilarity, respectively (Figure 1 and Supplementary Table 3).

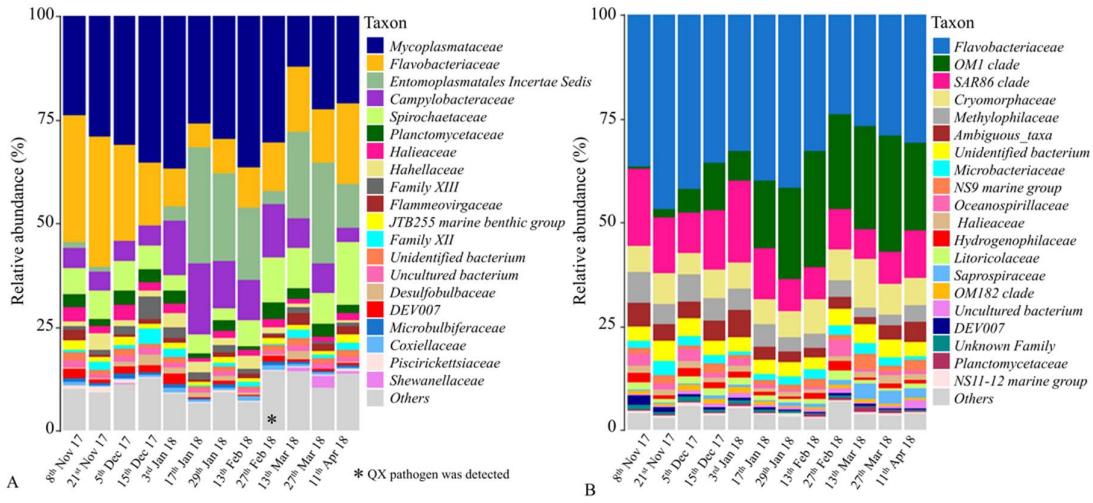


Figure 1: Microbiota of SRO (A) and water samples (B) showing the top 20 dominant and remaining taxa in 12 sampling time points. Data is summarised at the family level. The asterisk in A indicates the first detection of the QX pathogen.

3.3. SRO associated bacterial communities differ according to presence of the QX pathogen

Of the QX-positive SROs, the microbiota of SROs with and without sporonts did not significantly differ ($F = 0.9619$, $p = 0.5099$). Therefore, we examined whether the SRO

294 microbiota differed according to *M. sydneyi* parasite presence by comparing the Pre-QX, QX-
295 positive and QX-negative groups. Alpha diversity indices did not significantly differ between
296 these groups (Figure 2 and Supplementary Table 4), except for species richness in QX-positive
297 SROs, which was significantly lower than Pre-QX SROs (Kruskal-Wallis test, $H = 6.928$, $p =$
298 0.0085). An nMDS plot showed that the microbiota in QX-positive SROs was more tightly
299 clustered than the Pre-QX and QX-negative SRO microbiota (Supplementary Figure 4). These
300 patterns were confirmed by PERMANOVA with the microbiota associated with QX-positive
301 SROs significantly different to Pre-QX ($F = 9.423$, $p < 0.001$) and QX-negative SROs ($F =$
302 3.282, $p < 0.001$). Additionally, the microbiota of Pre-QX SROs differed to QX-negative SROs
303 ($F = 4.868$, $p < 0.001$).
304
305 SIMPER analysis revealed that the microbiota associated with QX-positive SROs was 86.5 %
306 and 84.9 % dissimilar to Pre-QX and QX-negative oysters respectively. Among the QX-
307 positive group, an OTU belonging to an unclassified species of the *Candidatus Hepatoplasma*
308 genus (OTU 11357) was substantially over-represented in comparison to Pre-QX and QX-
309 negative SROs, driving 14.5 % and 15.6 % of the dissimilarity respectively (Figure 3 and,
310 Supplementary Tables 5 and 6). An OTU belonging to an unclassified species of the *Borrelia*
311 genus (OTU 1) was also over-represented in the QX-positive group responsible for 7.5 % and
312 8.1 % of the dissimilarity when compared to the Pre-QX and QX-negative groups, respectively.
313 In contrast, an OTU assigned to an unclassified species of the *Mycoplasma* genus (OTU 11355)
314 was over-represented in the Pre-QX and QX-negative groups contributing 12.5 % and 12.9 %
315 of the dissimilarity respectively (Figure 3 and, Supplementary Tables 5 and 6).
316
317 A Welch's t-test (STAMP; Parks et al. 2014) identified 175 OTUs with statistically different
318 relative abundances between the Pre-QX and QX-infected groups. Of these, 14 OTUs varied
319 by at least 1 % relative abundance (Figure 4A). In comparisons between the QX-infected and
320 uninfected groups, this approach identified 23 OTUs that differed in relative abundance, with
321 7 OTUs varying by at least 1 % relative abundance (Figure 4B). Consistent with the SIMPER
322 results, an OTU assigned to an unclassified species of the *Mycoplasma* genus (OTU 11355)
323 was significantly overly represented in the Pre-QX and QX-negative groups relative to the QX-
324 positive group ($p < 0.001$ and $p = 0.001$ respectively). An OTU assigned to an unclassified
325 species of the *Borrelia* genus (OTU 1) was significantly higher in the QX-positive group
326 compared to the Pre-QX and QX-negative groups ($p < 0.001$ and $p < 0.001$, respectively).
327 Additionally, a member of the *Candidatus Endoecteinascidia* genus (OTU 10028) was

significantly higher in the QX-positive group compared to the Pre-QX and QX-negative groups ($p = 0.046$ and $p = 0.048$, respectively).

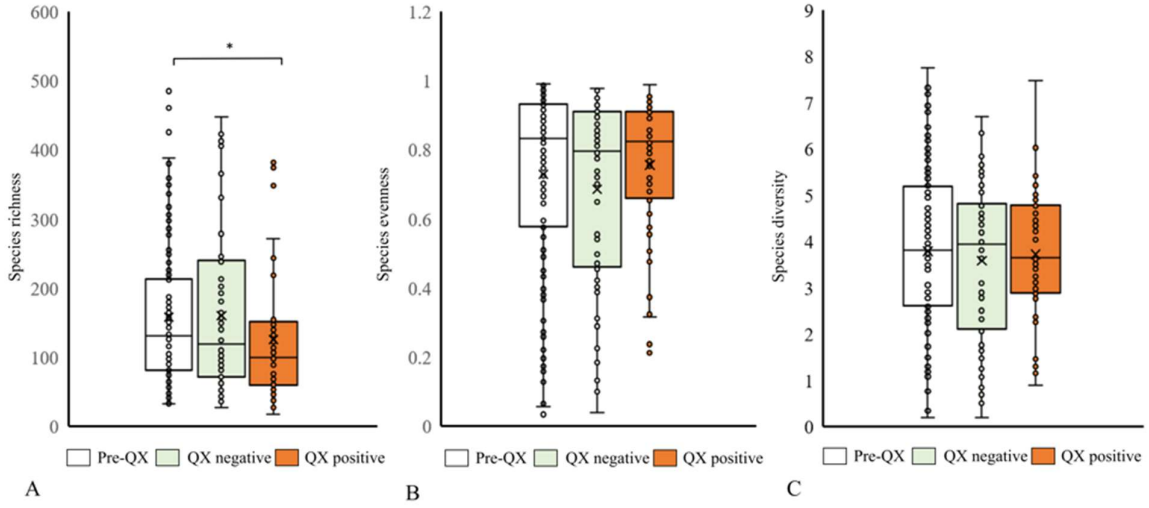


Figure 2: Box and whisker plot of species richness (A), evenness (B) and diversity (C) for Pre-QX, QX-negative and QX-positive SROs, (x) represents the mean of the data set. The asterisk indicates statistical significance at $p < 0.01$.

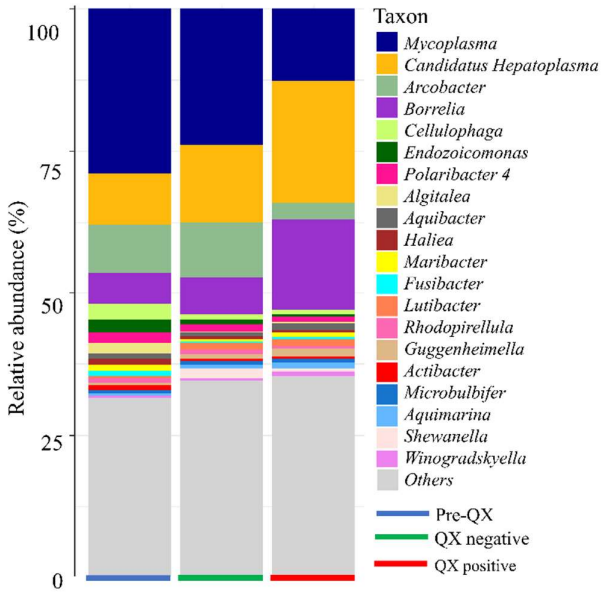


Figure 3: Microbiota composition of SRO groups showing the top 20 dominant and remaining taxa in Pre-QX (underlined by the blue bar), QX-negative (underlined by the green bar) and QX-positive (underlined by the red bar). Data is summarised at the genus level.

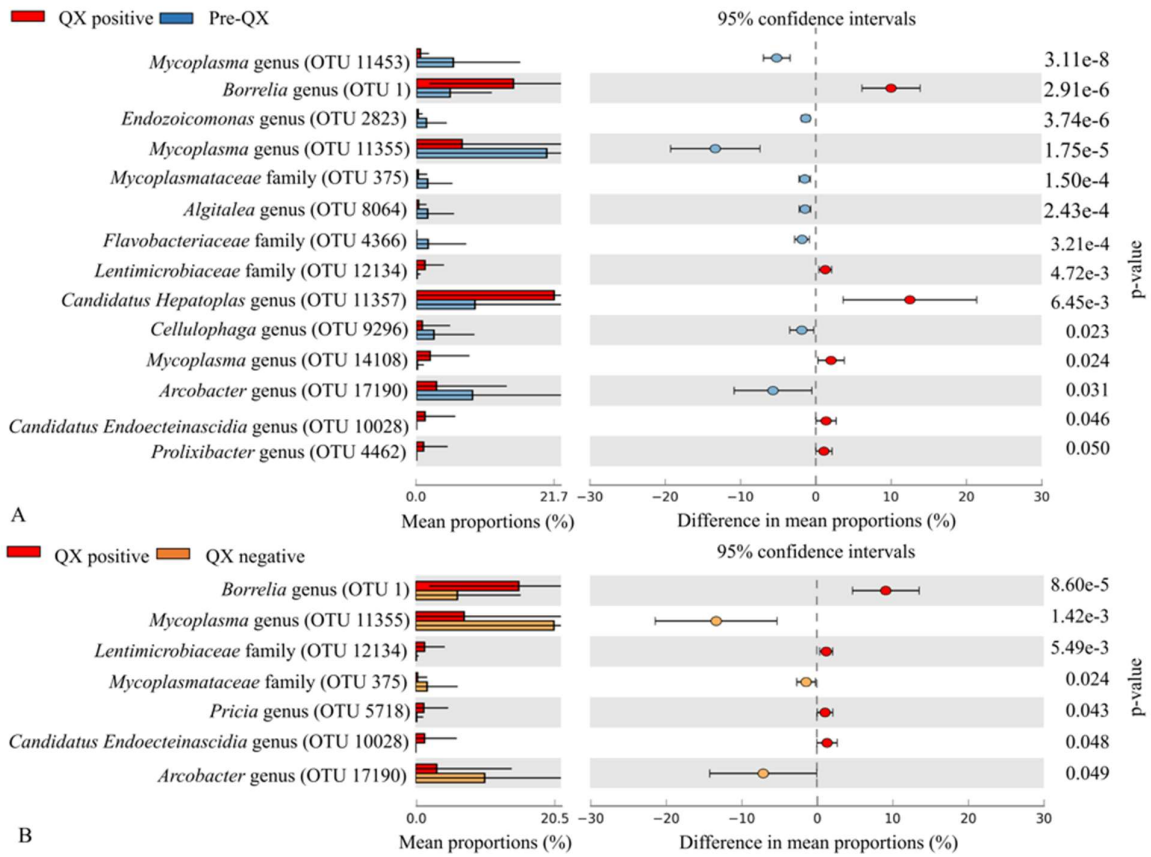


Figure 4: Extended error bar plot showing OTUs with a significant difference in relative abundance between the QX-positive and, Pre-QX (A) and QX-negative (B) groups.

3.4. Correlation between environmental variables, *M. sydneyi* and the SRO microbiota

We investigated correlations between measured environmental variables (Table 1) and the SRO microbiota to the presence of *M. sydneyi* in the SROs (Figure 5A). Phosphate displayed the strongest positive correlation with *M. sydneyi* (SpearmanRho = 0.256 and $p < 0.001$) and pH exhibited the strongest negative correlation (SpearmanRho = -0.24 and $p < 0.001$). *Marteilia sydneyi* was negatively correlated with OTUs assigned to unclassified species of the *Mycoplasma* (OTU 11355) (SpearmanRho = -0.33 and $p < 0.001$) and *Polaribacter* (OTU 9546; SpearmanRho = -0.15 and $p < 0.001$) genera (Fig. 5B and Supplementary Table 7) and, was positively correlated with OTUs assigned to unclassified species of the *Borrelia* (OTU 1) (SpearmanRho = 0.38 and $p < 0.001$) and *Candidatus Hepatoplasma* (OTU 11357; SpearmanRho = 0.31 and $p < 0.001$) genera (Fig. 5B and Supplementary Table 7). The

correlations between presence of *M. sydneyi* and OTUs assigned to unclassified species of the *Borrelia* (OTU 1) and *Mycoplasma* (OTU 11355) genera were stronger than to phosphate and pH (Supplementary Table 7). Of the dominant OTUs, phosphate displayed a negative correlation with a member of the *Mycoplasma* genus (OTU 11355) (SpearmanRho = -0.13 and $p < 0.001$) and a positive correlation with a member of the *Candidatus Hepatoplasma* genus (OTU 11357; SpearmanRho = 0.48 and $p < 0.001$ (Figure 5B).

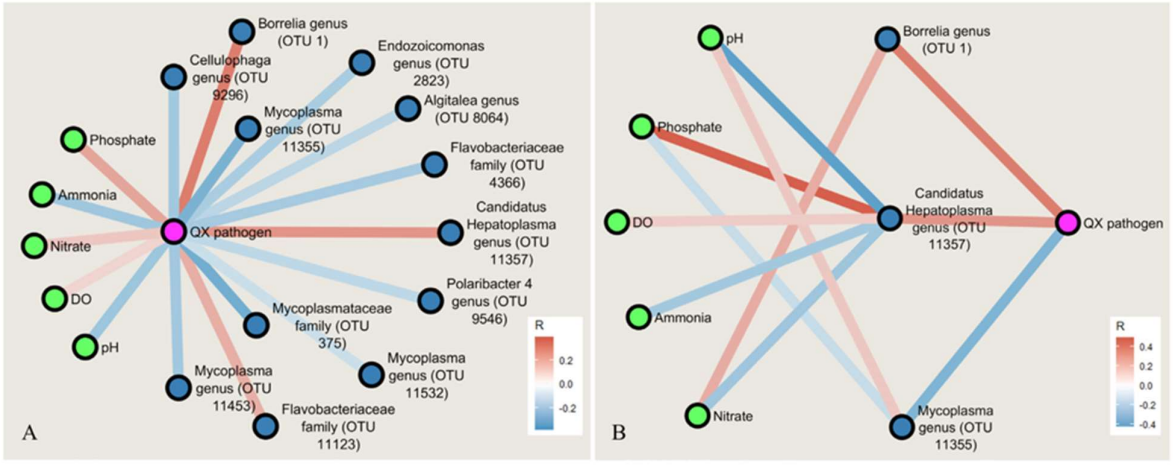


Figure 5: Network analysis showing significant correlations of the QX pathogen (*Marteilia sydneyi)* with specific OTUs and with measured environmental variables (A). Correlation of environmental variables and the *M. sydneyi* with three dominant OTUs (B). Blue and red lines represent negative and positive correlations respectively. The lines (edges) are coloured by Spearman correlations – the darker the colour, the stronger the correlation.

Table 1: Environmental variables at each sampling point throughout the study

Time	pH	DO (mg/L)	Temp. (^{0}C)	Conduct. (µS/cm)	Nitrate (mg/L)	Ammonia (mg/L)	Phosphate (mg/L)	Chlo. (µg/mL)	Rainfall*
8-Nov-17	9.8	8.7	19.9	50.5	0.027 ± 0.006	0.019 ± 0.009	0.012 ± 0.003	0.033 ± 0.013	Rainfall over 3 days occurred the day before sampling (5.13 mm/day)
21-Nov-17	7.9	8.9	20.3	32.9	0.037 ± 0.006	0.027 ± 0.008	0.009 ± 0.002	0.030 ± 0.07	Rainfall event on day of sampling (1.3 mm)
5-Dec-17	7.8	7.4	23.8	49.9	0.115 ± 0.069	0.029 ± 0.005	0.0177 ± 0.012	0.138 ± 0.15	Rainfall over 3 days occurred 2 days before sampling (5.53 mm/day)
15-Dec-17	9.0	7.4	26.4	33.8	0.023 ± 0.027	0.023 ± 0.007	0.027 ± 0.002	0.024 ± 0.006	Rainfall a day occurred 5 days before sampling (5.4mm)
3-Jan-18	8.1	7.3	26	35.3	0.004 ± 0.00	0.013 ± 0.002	0.026 ± 0.003	0.0290 ± 0.004	Rainfall a day occurred 3 days before sampling (3mm) and event on day of sampling (1.5 mm)
17-Jan-18	7.9	7.9	23.7	52.2	0.005 ± 0.001	0.013 ± 0.002	0.027 ± 0.004	0.034 ± 0.02	Rainfall 2 days occurred one week before sampling (23 mm) and the day before sampling (1.5 mm)
29-Jan-18	8.0	7.1	27.2	52.5	0.005 ± 0.002	0.012 ± 0.002	0.038 ± 0.005	0.021 ± 0.01	Rainfall occurred over 2 days including day of sampling (1.0 mm/day)
13-Feb-18	8.0	7.7	27.9	52.5	0.006 ± 0.002	0.014 ± 0.004	0.038 ± 0.005	0.019 ± 0.003	Rainfall over 2 days occurred one day before sampling (accumulated total of 4.0 mm)
27-Feb-18^	7.9	7.0	24	46	0.013 ± 0.015	0.026 ± 0.029	0.050 ± 0.009	0.024 ± 0.005	Rainfall 2 events in 4 days occurred one week before sampling (accumulated total of 44.6 mm)
13-Mar-18	7.5	7.5	24.7	49.4	0.027 ± 0.006	0.040 ± 0.024	0.044 ± 0.014	0.030 ± 0.004	Rainfall 4 days occurred 2 days before sampling (accumulated total of 18.2 mm
27-Mar-18	7.7	8.0	22.9	47.5	0.011 ± 0.008	0.012 ± 0.007	0.035 ± 0.003	0.031 ± 0.003	Rainfall 4 days occurred 2 days before sampling (accumulated total of 27.8 mm
11-Apr-18	8.1	7.6	24.9	49.6	0.030 ± 0.01	0.012 ± 0.001	0.034 ± 0.002	0.024 ± 0.002	No

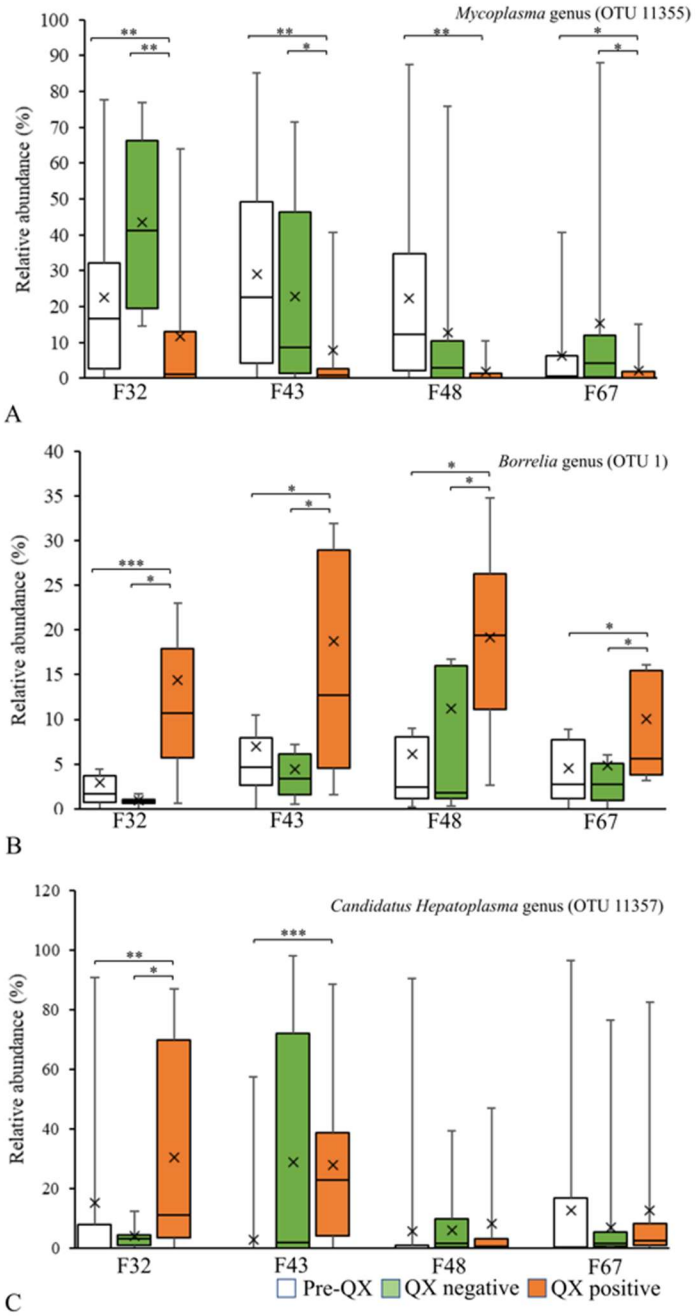
*Data obtain from (Bureau of Meteorology, 2019). DO: dissolved oxygen, Temp: temperature, Conduct: conductivity, Chlo: chlorophyll-a.

^First week QX was detected.

3.5. Variability of the SRO microbiota across families

We examined whether the SRO microbiota for the Pre-QX, QX-positive and QX-negative groups differed across the four families. Overall, alpha indices did not significantly differ between the families in each group (Kruskal-Wallis test, $p>0.05$; Supplementary Figure 5 and Supplementary Table 8), except in the QX-infected group where F32 and F67 were significantly different in species diversity (Kruskal-Wallis test, $H = 6.961$, $p = 0.0083$) and species evenness (Kruskal-Wallis test; $H = 5.25$, $p = 0.02498$) (Supplementary Figure 5 and Supplementary Table 8). No clear dissimilarity of the microbiota composition between families was observed in a nMDS analysis (Supplementary Figure 6), however statistical analyses identified differences between the SRO microbiota of most of the families in each group (Supplementary Table 9).

Given the variability in the SRO microbiota across families, we focused on the main taxa driving the dissimilarity between the groups, namely OTUs assigned to unclassified species of the *Mycoplasma* (OTU 11355), *Borrelia* (OTU 1) and *Candidatus Hepatoplasma* (OTU 11357) genera. Our data showed that the relative abundance of OTU 11355 from the *Mycoplasma* genus was higher in the Pre-QX and QX-negative groups compared to the QX-positive group for F32, F43 and F67 and higher in the Pre-QX group compared to QX-positive in F48 (Figure 6). The relative abundance of OTU 1 from the *Borrelia* genus was higher in the QX-positive group compared to the other two groups in all families (Figure 6). OTU 11357 from the *Candidatus Hepatoplasma* genus was more variable across families and was higher in the QX-positive group compared to Pre-QX in F32 and F43 but not the other families. In F32, it was higher in the QX-positive group compared to the QX-negative group (Figure 6).



394

395 **Figure 6:** Box and whisker plot showing relative abundance of OTUs assigned to the (A)
396 *Mycoplasma* (OTU 11355), (B) *Borrelia* (OTU 1) and (C) *Candidatus Hepatoplasma* (OTU
397 11357) genera. The x represents the mean of the data set. The single, double and triple asterisks
398 indicate statistical significance at $p < 0.05$, < 0.01 and < 0.001 respectively.

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400

4. Discussion

This study characterised the SRO microbiota before and during a QX disease event. Consistent with previous studies in the Pacific oyster (Lokmer *et al.*, 2016b) and the SRO (Nguyen *et al.*, 2020), our results indicate that the SRO microbiota is highly distinct from bacteria in the surrounding seawater. Additionally, the role of SRO genetics in microbiota structure in our prior study (Nguyen *et al.*, 2020) was confirmed with families showing differences in microbiota despite all having a similar phenotype with respect to QX disease susceptibility. Over the study period, the SRO microbiota was dominated by members of the *Mycoplasma, Candidatus Hepatoplasma, Arcobacter* and *Borrelia* genera. These findings are also consistent with our previous SRO microbiota observations in two other locations and over two seasons (Nguyen *et al.*, 2020). Of these genera, the *Mycoplasma* genus was the most relatively abundant, which is also consistent with other studies that have shown significant abundance of this genera in healthy Eastern oysters (King *et al.*, 2012), Pacific oysters (Wegner *et al.*, 2013; King *et al.*, 2019a; King *et al.*, 2019c) and SROs (Green & Barnes, 2010; Nguyen *et al.*, 2020). Temporal heterogeneity in the oyster microbiota composition has previously been observed (Lokmer *et al.*, 2016a; Pierce *et al.*, 2016; Pierce & Ward, 2019) and was also observed here with a relative decline in *Mycoplasma* and a relative increase in OTUs assigned to the *Candidatus Hepatoplasma* and *Borrelia* genera in the QX-negative group when compared to the Pre-QX group, correlating with SROs sampled during the Austral early autumn and summer seasons, respectively.

We observed that the microbiota of QX-positive SROs was different to Pre-QX and QX-negative SROs, although no difference was observed between QX-positive SROs with and without sporonts indicating that the microbiota shift in QX-positive SROs occurs shortly before or shortly after *M. sydneyi* colonisation. In our study, the QX-positive microbiota appeared to become more tightly clustered than the Pre-QX and QX-negative bacterial communities and had reduced species richness. The reason for this is unknown but parasitic infections reduce the filter-feeding capacity of mussels (Stier *et al.*, 2015) and this is likely also occurring in QX infected SROs as judged by their reduced growth (Nell & Perkins, 2006; Dove *et al.*, 2013) resulting in reduced allochthonous input of bacteria and a possible decrease in species richness. Microbiota differences according to presence of the QX pathogen were mainly driven by changes in the relative abundance of OTUs assigned to unclassified species of the *Mycoplasma, Candidatus Hepatoplasma* and *Borrelia*

genera however, shifts in the *Candidatus Hepatoplasma* OTU were not consistent across the four families. An OTU assigned to the *Mycoplasma* genus (OTU 11355) was significantly lower in the QX-positive group when compared to Pre-QX and QX-negative groups. We have previously observed that the relative abundance of *Mycoplasma* OTUs is higher in oysters bred for QX resistance compared to QX sensitive oysters in winter but not summer (Nguyen *et al.*, 2020). As *Mycoplasma* species are consistently found to be dominant in healthy Eastern oysters (King *et al.*, 2012), Pacific oysters (Wegner *et al.*, 2013; King *et al.*, 2019a; King *et al.*, 2019c) and SROs (Green & Barnes, 2010; Nguyen *et al.*, 2020), bacteria from this genus may have an important role in oyster health. We hypothesise that relative abundance decreases in the overall *Mycoplasma* genus or of a specific OTU(s) could be an indication of, or an increased susceptibility to, disease. Here, we observed a relative decrease in a specific *Mycoplasma* OTU in QX-negative oysters during the QX disease event compared to the Pre-QX oysters with a further relative decrease in SROs that were QX-positive. Our data may suggest that a threshold reduction of this *Mycoplasma* OTU, which in our study positively correlated with phosphate, may be required for QX infection to occur rather than facilitating disease progression and may explain why QX resistant oysters are protected (Nguyen *et al.*, 2020). Alternatively, a relative decrease in this *Mycoplasma* OTU is simply a signature of a stressed host that is becoming, or has become, susceptible to QX infection.

In addition to the decrease in the relative abundance of a *Mycoplasma* OTU, increases in the relative abundance of OTUs identified as members of the *Candidatus Hepatoplasma* and *Borrelia* genera were observed in the microbiota of QX-positive oysters. *Candidatus Hepatoplasma* positively correlated with phosphate and negatively correlated with pH whereas, *Borrelia* positively correlated with nitrate. As was observed with the *Mycoplasma* OTU, increases in the relative abundance of these OTUs occurred in the QX-negative SROs when compared to the Pre-QX SROs. This pattern may indicate a progressive replacement of the *Mycoplasma* OTU with bacteria from these genera. *Candidatus Hepatoplasma* is commonly found in other marine organisms such as starfish, lobsters, corals (Meziti *et al.*, 2012; Nakagawa *et al.*, 2017; van de Water *et al.*, 2018) and SROs (Nguyen *et al.*, 2020) however, their function role, if any, is currently unknown. In our previous study, a member belonging to the *Candidatus Hepatoplasma* genus was relatively more abundant in QX sensitive oysters compared to QX resistant oysters at one of two locations investigated, in winter but not summer (Nguyen *et al.*, 2020). This may indicate *Candidatus Hepatoplasma* is important in QX disease or that its increase is a signature of QX disease or susceptibility however, our study showed that shifts in this OTU were not consistent across

families. On the other hand, *Borrelia* (OTU 1) was consistently over-represented in QX-positive SROs compared to those from the Pre-QX and QX-negative groups. Interestingly, our previous study found an OTU belonging to *Borrelia* as over-represented in QX resistant oysters, whereas in this study, *Borrelia* was associated with QX-positive oysters (Nguyen *et al.*, 2020). These *Borrelia* OTUs are not identical and could be fulfilling different roles. *Borrelia* belongs to the Spirochaete phylum and has been detected in the crystalline styles (non-cellular cylindrical rods of a gelatinous texture found in digestive systems) of Pacific Oysters (Husmann *et al.*, 2010) as well as the digestive gland of healthy SROs (Green & Barnes, 2010) and sequences belonging to this phylum were recently identified as members of the core Pacific Oyster microbiota (King *et al.*, 2020). Notably, a bacterium belonging to the Spirochaete phylum has been implicated as the causative agents of Pearl Oyster disease (Matsuyama et al. 2017). As with the *Mycoplasma* OTU, whether the relative increase in OTUs belonging to the *Candidatus Hepatoplasma* or *Borrelia* genera facilitates QX infection or their increase is a signature of QX susceptibility requires further investigation.

5. Conclusion

There is increasing evidence that the oyster microbiota can play a role in oyster disease, but the impact of the SRO microbiota on QX disease is yet to be resolved. Observing shifts in the microbiota before and during a disease event is essential when attempting to interpret the interplay between disease, the environment and the host microbiota. This study has revealed that the microbiota associated with QX-positive oysters are different from those of Pre-QX and QX-negative oysters. Microbiota variations were mainly driven by the relative abundance changes of several key OTUs belonging to the *Mycoplasma, Candidatus Hepatoplasma* and *Borrelia* genera, indicating possible roles for these bacteria in QX susceptibility. Additionally, this study has revealed that the microbiota of SROs with and without sporonts did not differ, implying that the observed shifts in microbiota occur shortly before or at the early stages of infection. This data will aid understanding of the potential involvement of the SRO microbiota during QX disease and may identify specific bacterial groups that may be useful for monitoring SRO health or identifying QX resistant SRO families from breeding programs.

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