Characterisation of the Sydney Rock Oyster microbiota and its association with QX disease

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A thesis submitted in fulfillment of the requirements for the degree: Doctor of Philosophy

University of Technology Sydney
School of Life Sciences
Climate Change Cluster

February 2021

Certificate of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the collaborative doctoral degree and/or fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This research was supported by an Australian Government Research Training Program.

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Acknowledgements

First and foremost, I would like to thank my principal advisor, Associate Professor Maurizio Labbate, for his constant guidance, encouragement, and patience throughout the entirety of my research. His guidance helped me in the research and writing in this thesis. This thesis would have been not possible without his help, exceptional supervision, and valuable contributions. I also would like to thank my co-advisors, Professor Justin Seymour, Dr. Wayne O'Connor and Dr. Michael Dove for their support, insightful comments and encouragement for which it would not have been possible to conduct this research. I also would like to thank my co-advisor Dr. Cheryl Jenkins for her support and guidance helping me in the QX disease diagnosis assays.

I would like to thank Dr. William King, Dr. Nahshon Siboni and Dr. Khandaker Rayhan Mahbub for helping me in field work and in the laboratory and for guiding my data analyses. I could not have been able to complete my research and data analyses without their help. I would like to thank the members of Associate Professor Maurizio Labbate and Professor Justin Seymour's laboratories for their help in the field and various laboratory work. I also would like to thank the UTS Graduate Research School for their help in administrative matters. Finally, I would like to acknowledge my family (especially my parents, my wife Huong Lan, and our son Duc Nguyen and our daughter Linh Phuong) and friends for all their love, encouragement and help getting me through one of the toughest times of my life.

Publications

Nguyen, V. K., King, W. L., Siboni, N., Mahbub, K. R., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). The Sydney rock oyster microbiota is influenced by location, season and genetics. *Aquaculture*, 735472. doi:https://doi.org/10.1016/j.aquaculture.2020.735472

Nguyen, V. K. King, W. L., Siboni, N., Mahbub, K. R., Rahman, Md. H., Jenkins, C., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). Dynamics of the Sydney rock oyster microbiome prior to and during a QX disease event. (Submitted)

Conference Presentations

Nguyen, V. K., King, W. L., Siboni, N., Mahbub, K. R., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2019)**.** Presentation title: The Sydney rock oyster microbiota is influenced by local environmental parameters and QX disease resistance. The international on Fish and Shellfish immunology, June 16-20, 2019 at Gran Canaria, Spain.

Nguyen, V.K. King, W.L., Siboni, N., Mahbub, K.R., Rahman, Md. H., Jenkins, C., Dove, M., O'Connor, W., Seymour, J.R., & Labbate, M. (2019). Poster**:** Variability of the Sydney rock oyster microbiome prior to and during a QX disease event. The 2019 NSW Oyster conference, August 6th -8th 2019 at Foster, NSW, Australia.

Abstract

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is native to Australia and is the most intensively farmed oyster species in the country. However, diseases such as Queensland unknown (QX) disease have caused substantial losses and impeded productivity. The aetiological agent of QX disease is the parasite *Marteilia sydneyi* causing disease seasonally, generally in the late summer and early autumn. QX infection initiates at the palps and gills before migrating into the digestive gland where sporulation occurs causing blockage and eventual starvation and death in the oyster. Emerging evidence suggests that QX disease outbreaks are driven by a series of complex environmental and host factors such as salinity and oyster genetics. To mitigate the impacts of QX disease, the New South Wales Department of Primary Industries (NSW DPI) has led a selective breeding program using both mass selection methods and family-based breeding to create lines that are resistant. Some families have shown approximately 85% survival through one cycle of disease however, the mechanisms that drive this resistance are poorly understood. One potential factor influencing QX disease resistance is the microbiota which is investigated in this thesis.

In other oyster species, the microbiota is emerging as a key factor in disease dynamics and studies have begun to unravel the environmental factors that influence its structure such as location, season, genetics and disease state. For the SRO, there is a paucity of studies examining the microbiota and the factors that influence the SRO microbiota composition. Therefore, this thesis aimed to explore the influence of geographic location, season (comparing summer and winter) and genetics (selectively bred SROs with a range of resistance to QX disease) on the SRO microbiota (Chapter 2). It also investigated microbiota dynamics prior to and during a QX disease event (Chapter 3) and, compared the SRO microbiota across families for

identifying taxa that could be used as indicators for healthy and QX-infected oysters (Chapter 4).

Overall, this thesis provides new knowledge of the SRO microbiota and the factors that influence the SRO microbiota assemblage and contributes valuable information on how the SRO microbiota shifts during a QX disease event and identifies putative key taxa that may be important for health and disease states and/or good indicator taxa of these states. A better understanding of the SRO microbiota, its potential involvement in health and disease may enable effective disease prevention in the future.

Abbreviations

SRO: Sydney rock oyster

C. gigas: *Crassostrea gigas* (also colloquially called the Pacific oyster)

S. glomerata: *Saccostrea glomerata* (also colloquially called the Sydney rock oyster)

O. edulis: *Ostrea edulis* (also colloquially called the European flat oyster)

C. virginica: Crassostrea virginica (also colloquially called the Eastern oyster)

OsHV-1: Ostreid herpes virus 1

OsHV-1 µvar: Ostreid herpes virus 1 microvariant

QX: Queensland Unknown

WM: Winter mortality

DPI: Department of Primary Industries

NCBI: National Center for Biotechnology Information

NSW: New South Wales

SRA: Sequence Read Archive

QIIME: Quantitative Insights Into Microbial Ecology

nMDS: Non-metric multidimensional scaling analysis

PERMANOVA: Permutational multivariate analysis of variance

SIMPER: Analysis of similarity percentages

STAMP: Statistical Analysis of Metagenomic Profiles

SRO: Sydney rock oyster

PCR: Polymerase chain reaction

qPCR: Quantitative Polymerase Chain Reaction

ISH: *in situ* hybridization

PO: Phenoloxidase

OTU: Operational taxonomic unit

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

µL: Microlitre

µM: Micromolar

km: Kilometre

L: Litre

bp: Base pair

mg: Milligrams

ng: Nanograms

Chapter 1 Literature review

1.1 Introduction

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is endemic to Australia and is a popular seafood product (Hussain *et al.*, 2017; Kow *et al.*, 2008; Liu *et al.*, 2006). SROs have been cultivated since the 1870s in New South Wales however, the production of this species has declined steadily since the mid-1970s. Diseases such Queensland unknown (QX) and winter mortality are major factors in the decline of SRO cultivation (Schrobback *et al.*, 2014). This thesis will focus on investigating the microbiota of the SRO and interrogate its possible role in QX disease susceptibility. To prepare the reader for the data in this thesis, this chapter will provide an overview of the SRO including its distribution and growth cycle, factors affecting its survival and growth including diseases, selective breeding methods for producing disease resistant families and, the microbiota of oysters.

1.1.1 Sydney rock oyster – distribution, growth cycle and anatomy

The SRO is a native species in Australia with a long history of culture (Schrobback *et al.*, 2014) with most SRO populations occurring along Australia's eastern coastline, including estuaries in New South Wales (NSW) and south eastern Queensland (Schrobback *et al.*, 2014). Intertidal estuarine environments including rocky habitats and mangrove forests are recognized as the natural habitats for the SRO however, they are also found on natural subtidal dredge beds (FAO, 2005).

The SRO is a bisexual aquatic animal meaning their sex changes during their life. Individuals usually begin as males at the first spawning (Dinamani, 1974) therefore, the percentage of males among younger oysters (one year or less than one year in age) is generally higher than in that of older oysters (Roughley, 1933). The main spawning season of the SRO is from

November to May (Dove & O'Connor, 2012) with one SRO female capable of producing up to 20 million eggs (O'Connor *et al.*, 2008). After eggs are fertilized by sperm in the water column, they proceed through a series of larval developmental stages before developing into small oysters called spat (Figure 1-1) (King *et al.*, 2019a; O'Connor *et al.*, 2008; Wallace *et al.*, 2008). The trochophore stage occurs around 6 hours after fertilization and is a free-swimming larval stage. Normally, the trochophore stage lasts about 12 - 14 hours before development to the veliger stage (also called the D-stage) which is able to ingest algae (Helm *et al.*, 2004; O'Connor *et al.*, 2008). The pediveliger stage appears approximately 18 days after spawning and consists of a veliger with a foot allowing it to crawl along surfaces (O'Connor *et al.*, 2008). The foot helps the pediveliger find a suitable substrate for final settlement before developing into spat (Wallace *et al.*, 2008), that take approximately 2 - 3 years to grow to marketable size (O'Connor *et al.*, 2014).

Generally, the anatomy of the oyster is not variable between species (Evseev *et al.*, 1996; Galtsoff, 1964; Thomson, 1954) and a general structure is shown in Figure 1-2. As other bivalve species, oysters have two shell valves which act as a skeleton for the attachment of the adductor muscle and to protect oyster against predators (Gosling, 2015). Surrounding the soft organ system is the mantle which has many different functions such as protection of organs, aids inflow of water and directs food particles onto the gills (Galtsoff, 1964; Gosling, 2015). The gills have a respiratory role and filter water to collect food particles which move to the palps where sorting occurs before food particles enter the digestive system (Galtsoff, 1964; Gosling, 2015; Kennedy *et al.*, 1996). The digestive gland consists of a large number of compact tubules (Galtsoff, 1964) containing digestive enzymes which are utilised by the stomach for food digestion (Gosling, 2015).

The adductor muscle is a large organ located in the posterior region of the body (Kennedy *et al.*, 1996). This muscle controls opening and closing of the shell (Zhu *et al.*, 2016) and contains large haemolymph sinuses (Gagnaire *et al.*, 2008). Haemolymph sinuses ramify throughout major organ systems and is the main haemolymph supply for the gills where then it returns to the heart for circulation (Bayne, 2017).

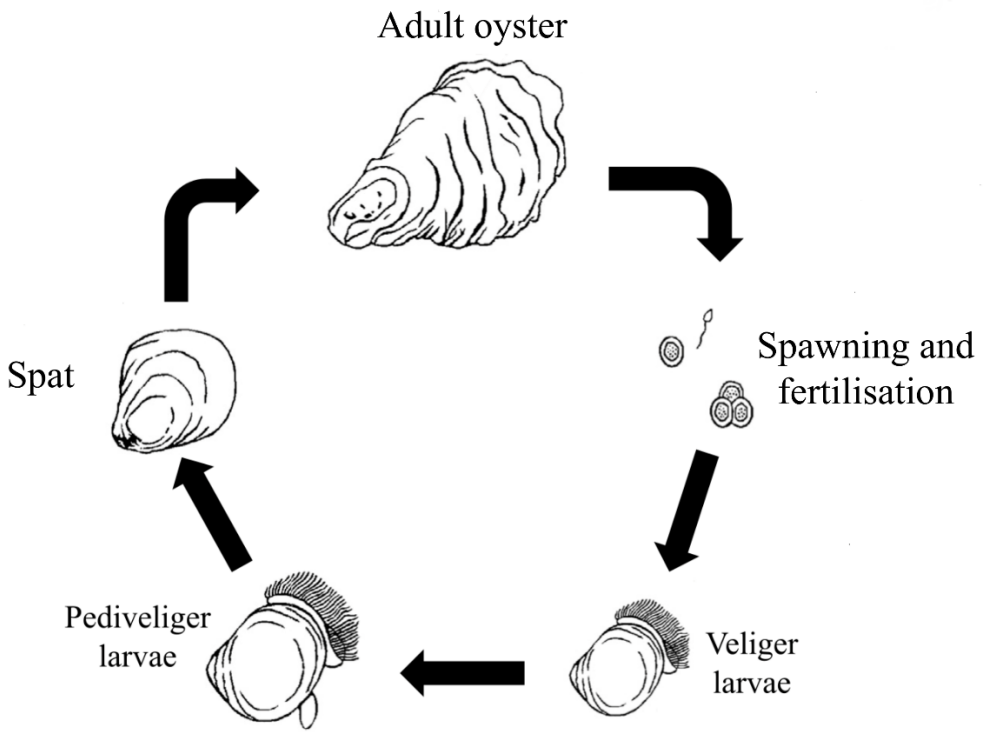

Figure 1-1: The general life cycle of an oyster. Trochophore stage occurs around 6 hours after fertilization and this stage takes 12 - 14 hours at 26°C before development to the Veliger (Helm *et al.*, 2004; O'Connor *et al.*, 2008). Then, the Pediveliger stage appears about 18 days at 26°C after spawning and metamorphose into to small oysters called spat that take around 2- 3 years to grow to marketable size (O'Connor *et al.*, 2014; Wallace *et al.*, 2008). Adapted with permission from Elsevier (King *et al.*, 2019a).

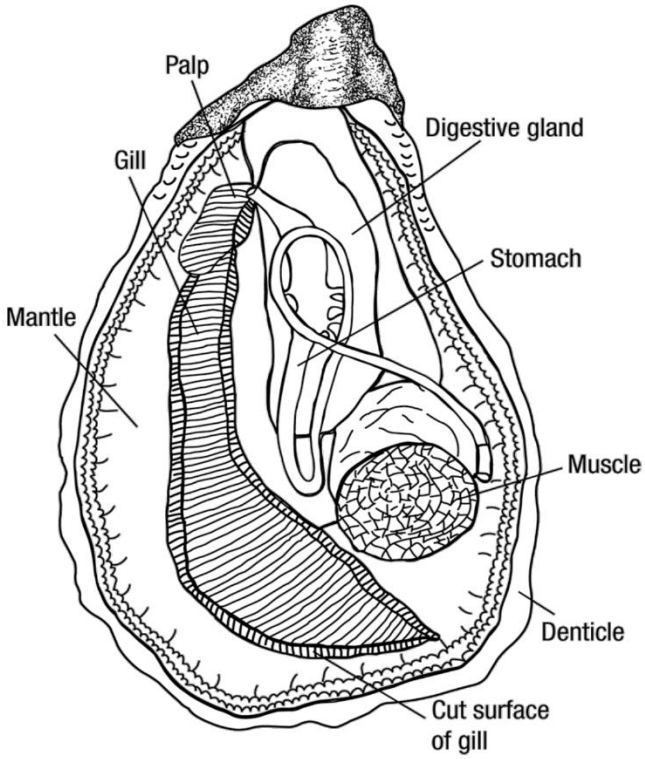

Figure 1-2: The anatomy of an oyster. Reproduced with permission from the Australian Government, Department of Agriculture, Water and the Environment (Department of Agriculture, Water and the Environment, 2020).

1.1.2 The Sydney rock oyster industry

The Sydney rock oyster is one of the most intensively cultivated oyster species in Australia (O'Connor & Dove, 2009; Schrobback *et al.*, 2014). The historical development of the SRO industry can be divided into five stages (Figure 1-3), including pre – European settlement, early commercialization, gradual expansion, growth and maturity and consolidation (Schrobback *et al.*, 2014). At the first stage (from Pre-European settlement to before 1788), oysters were collected and consumed by aboriginal people. In the second early commercialisation stage (around 1790s to early 1900s), large scale gathering of oysters occurred by hand picking from

beds and banks in the intertidal zone and using a dredging basket from a boat to collect oysters from 4 meters below the water line. Additionally, farmers used sticks, rocks and shell for oyster collecting and growing for human consumption (Nell, 1993; Roughley, 1922). In the gradual expansion stage (1910s to 1950s), the stick culture method was introduced which involves collecting oyster larvae using bundled sticks that are then separated and inserted into the ground to grow oysters to market size (Roughley, 1922). Consequently, oyster production increased (Schrobback et al., 2014). In the growth and maturity stage (1960s to early 1980s), the SRO industry developed significantly reaching peak production as a result of improvement of production methods and increased culture area (Schrobback et al., 2014). In the last consolidation stage (mid-1980s to present), the number of oyster growers and production volume has decreased due to several issues such as disease (such as QX and winter mortality – described in section 1.1.4) and environmental degradation leading to increased research and development into SRO disease such as breeding programs that enhance SRO disease resistance (Schrobback et al., 2014).

Because of improved farming methods, the production volume of the SRO industry increased rapidly during the first part of the 20th century, and production output peaked in the late 1970s with this industry leading aquaculture production in Australia until the early 1990s (Schrobback *et al.*, 2014). Production output peaked at 17 million dozen oysters in 1976/1977 (Department of Primary Industries, 2016). However, since the mid-1970's, production of this species has been impacted by disease and environmental degradation (Schrobback *et al.*, 2014), particular QX disease, which can recurrently cause up to 90% mortality in affected estuaries (Department of Primary Industries, 2016; Nell, 2007; O'Connor & Dove, 2009; Peters & Raftos, 2003; Schrobback *et al.*, 2014), and has since declined to about 4.55 million dozen oysters in 2011/2012 (Livingstone, 2013). Currently, the production output of SRO in New

South Wales is approximately 6 million dozen (Jefferson, 2019) with a value of about $48.74 million AUD and accounting for 89% of aquaculture output in this state (Jefferson, 2019).

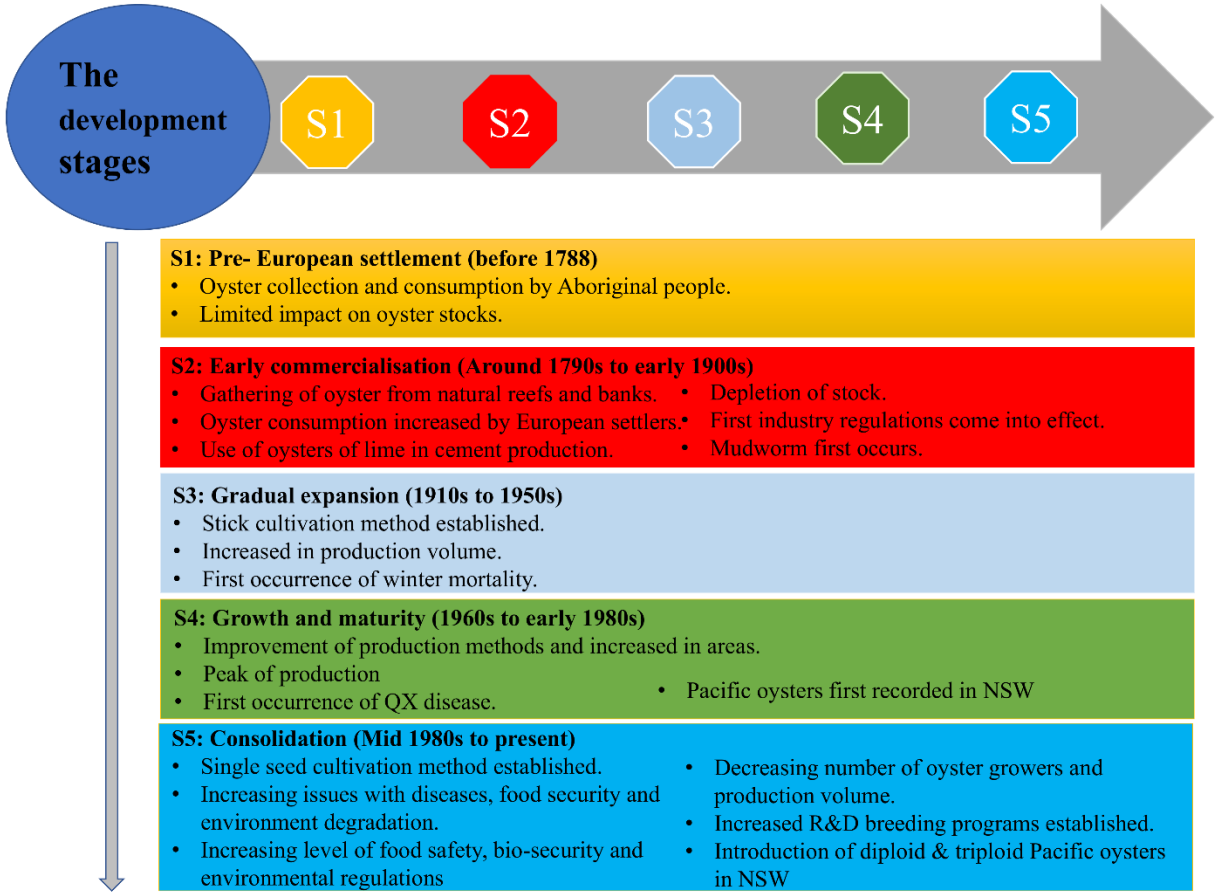

Figure 1-3: The developmental stages of the Sydney rock oyster industry in Australia. The Pre-European settlement stage consisted of consumption by indigenous aboriginals with consumption increasing upon European settlement and subsequent commercialisation and increased scale of production. Disease and environmental degradation have decreased production from its peak reached in the 1980's. Image originally produced using information from Schrobback *et al.* (2014).

1.1.3 Factors affecting the survival and growth of Sydney rock oysters

Environmental parameters affect the physiology and development of oysters as well as disease outbreaks (Shumway, 1996). Salinity and temperature are the main factors that influence the early stages of SRO development and survival (Dove & O'connor, 2007; Nell & Holliday, 1988). The optimal salinity and temperature for growth and survival of SRO larvae occurs within the ranges of 27 – 39 ppt (part per thousand) and 26 – 30^0C respectively (Dove & O'connor, 2007; Nell & Holliday, 1988), with an optimal salinity and temperature of 35 ppt and 30^0C when the SRO reach the spat stage (Dove & O'connor, 2007). A decrease in water pH has been demonstrated to have adverse impacts on SRO larval development, growth and survival (Dove & Sammut, 2007b; Parker *et al.*, 2009; Parker *et al.*, 2011; Wilson & Hyne, 1997). The filtration rate of SROs at pH 5.5 decreases significantly and the mantle and gill soft tissues of the oyster changes after short term exposure to acid sulfate soil-affected water (Dove & Sammut, 2007a). Specifically, these changes include the occurrence of necrosis of the gill filaments, necrosis and sloughing of the mantle epithelial cells in oysters (Dove & Sammut, 2007a). Other studies have shown that ocean acidification (as resulting of increasing pCO_2 levels) negatively impacts the development of SRO larvae, leading to slower progress, abnormalities and smaller sizes (Parker *et al.*, 2009). For example, increasing pCO_2 reduced 25% in shell growth of selectively bred SROs (Parker *et al.*, 2011). Additionally, declining water quality derived from anthropogenic disturbance including clearing of catchment for agriculture and urban development have negative effects on the survival and growth of SROs (Diggles, 2013; Gillies *et al.*, 2018; Paterson *et al.*, 2003) and environmental degradation is considered as a factor that affects the production output of SRO farms (Schrobback *et al.*, 2014). Besides environmental factors, growth of SRO can also be impacted by diseases including mudworm, flatworm, winter mortality and QX disease (Nell, 2007).

1.1.4 Sydney rock oyster diseases

1.1.4.1 Mudworm and flatworm

A parasitic worm from the polychaete family spionidae affecting SROs was first reported in the Hunter River, NSW in 1882 (Read, 2010). Since then, it has spread through estuaries in NSW and subsequently in Queensland (Ogburn *et al.*, 2007). Among all species, *Polydora websteri* is considered as the most damaging to the oyster (Nell, 2007; Read, 2010) and is commonly called mudworm (Nell, 2007). Mudworm larvae enter, live and grow inside of the oyster shell which causes slowing of growth, reduction in oyster health and in some case oyster mortality (Nell, 2007; Read, 2010). The occurrence of mudworm led to a decrease in SRO production of NSW in 1891 (Ogburn *et al.*, 2007). Mudworm can be controlled by the combination of drying and bathing the oysters in an iodine-based disinfectant solution (Nell, 2007; Nell, 2001). Besides mudworm, survival of SROs also is threatened by flatworm (*Imogine mcgrathi*) which have long been recognised as common predator of bivalves and oysters with each flatworm consuming about one oyster per month (Nell, 2007; O'Connor & Newman, 2001). Flatworm disease of SRO was first identified in NSW in the early 1890's (Nell, 2007; Nell, 2001). Exposure to freshwater for 5 mins is recognised as an effective method to kill the adult flatworms (Nell, 2007).

1.1.4.2 Winter Mortality disease

Winter mortality (WM) was first observed in 1923 in the Georges River (Nell, 2007; Roughley, 1926; Spiers *et al.*, 2014). The disease is found mainly in oysters that are 2 - 3 years old (Nell & Perkins, 2006; Wolf, 1967) with mortality rates sometimes exceeding 80 % (Nell, 2007). The first investigation into the cause of WM was in the Georges River, NSW between 1924 and 1925 (Roughley, 1926) where it was determined that cold temperature correlated with the

mortality (Roughley, 1926). In 1988, WM was observed in the Georges River and Woolooware Bay, NSW (Farley *et al.*, 1988) and since 2010, has been observed in many other NSW estuaries, from Pambula Lake in the south to Port Stephens in the north (Spiers *et al.*, 2014). Gross clinical signs of WM include the presence of pustules, abscesses, and ulcerations in different tissues such as the mantle, palps, gills, adductor muscle, gonad, digestive diverticulum and alimentary tract (Farley *et al.*, 1988; Roughley, 1926; Spiers *et al.*, 2014). However, winter mortality can also occur without gross clinical signs and histological changes characteristic of this disease (Spiers *et al.*, 2014) indicating that the mortality is unpredictable making sampling to study the disease challenging.

There has been considerable confusion related to the identification of a WM pathogen. Based on ultrastructural observations of a parasite within the SRO gill and digestive gland tissue, the pathogen of WM was first identified as the protozoan parasite *Mikrocytos roughleyi* (Farley *et al.*, 1988), which is small (2 – 3 μm) and it is difficult to diagnose from either tissue imprints or histological preparations (Adlard & Lester, 1995). Using molecular phylogenetic analyses of small-subunit ribosomal DNA (SSU rDNA) sequence, the pathogen was reclassified as *Bonamia roughleyi* (Cochennec *et al.*, 2003), a species closely related to *Bonamia exitiosa* and sharing an identical SSU rDNA sequences (Carnegie & Cochennec-Laureau, 2004).

A longitudinal study of WM was conducted from February to November 2010 in Georges and Shoalhaven Rivers (Spiers *et al.*, 2014). Although mortalities occurred in both study sites, gross clinical signs and histological changes characteristic of WM were only observed in oyster samples from Georges River, and PCR results showed a very low prevalence of *Bonamia* sp. relative to histological changes characterized by the microcell-like structures located within haemocytes (Spiers *et al.*, 2014). Therefore, it is still unclear whether *Bonamia* sp. is the sole

pathogen responsible for WM or whether more than one pathogen is responsible (Spiers *et al.*, 2014).

Winter mortality is dependent on environmental conditions, with high salinities and low temperatures increasing the likelihood of a severe WM event (Nell, 2007). Therefore, impacts of the mortality can be reduced by moving oysters further upstream to areas of lower salinity sites before May (Nell, 2007) and increasing the growing height to 150 mm above normal growing height (Nell, 2007; Smith & Longmore, 1980) or harvesting before winter (Nell, 2007). Furthermore, the SRO breeding program has improved resistance levels to this disease in selected stock. Mortality in fourth generation selected lines of SROs was only 23%, with 52% in control oysters when exposed to winter mortality (Dove *et al.*, 2013b).

1.1.4.3 QX disease

QX disease is a major disease impacting SRO culture in NSW and southern Queensland, causing annual losses of nearly 100% in some areas (Peters & Raftos, 2003). This disease was first detected in SRO from Moreton Bay, Queensland in the late 1960s (Wolf, 1972). Since the late 1970s, this disease has spread extensively in the south eastern Queensland through to the Macleay River on the mid-north coast of NSW (Adlard & Ernst, 1995) and has consistently been observed in several NSW estuaries (Nell, 2007; Raftos *et al.*, 2014). After the first significant case of this disease in northern NSW estuaries, production of SRO in NSW declined significantly. For example, within 26 years (from 1974/75 to 2000/01), SRO production in the Tweed, Richmond and Clarence Rivers was reduced by 56% (Nell, 2001). In particular, QX has resulted in the collapse of SRO culture in the Georges River in 1994 and Hawkesbury River in 2004 (Dove *et al.*, 2013b).

The pathogen responsible for QX disease was first described in 1976 and is a spore-forming protozoan parasite called *Marteilia sydneyi* (Perkins & Wolf, 1976). This pathogen is known to infect the SRO as a uninucleate stem cell and then undergoes developmental stages within the oyster as described in Figure 1-4 (Kleeman *et al.*, 2002). The parasite infiltrates the oyster through the palps and gills where extrasporogonic replication occurs in the epithelium resulting in four daughter cells contained within an enlarged stem cell produced from a daughter cell within a vacuole in the cytoplasm of the uninucleate stem cell by binary fission (Kleeman *et al.*, 2002). The stem cell degenerates to release the bicellular daughter cells which penetrate the basal membrane haemolymph spaces in the surrounding connective tissue resulting in the parasite being distributed systematically throughout the oyster. Nurse cells (infected cells) are established at the base of the epithelial cells in the digestive gland tubules and daughter cells are formed within the nurse cells. Consequently, all available sites within the digestive tubule are occupied by daughter cells. Each daughter cell internally cleaves a secondary cell, and the nurse cells degrades (Kleeman *et al.*, 2002). Sporulation proceeds in the digestive gland with 8 or 16 sporonts (an encysted spore) formed within a sporangiosorus causing a blockage and oysters slowly starve to death (Wolf, 1979). Each sporont has two spores and each spore has three concentric cells (Perkins and Wolf 1976). Finally, mature sporonts (Sp) containing two tricellular spores are shed into the tubule lumen (Kleeman *et al.*, 2002), and can be detected approximately 2 weeks after infection (Peters & Raftos, 2003). Sporonts are shed into the environment before oyster death (Roubal *et al.*, 1989) surviving for a relatively short period of 7 to 35 days, well below the 10 - 12 months infection cycle (Wesche *et al.*, 1999). As a result, an environmental reservoir for the pathogen is assumed with a recent study identifying this parasite in a polychaete worm (*Nephtys australiensis*) (Adlard & Nolan, 2015).

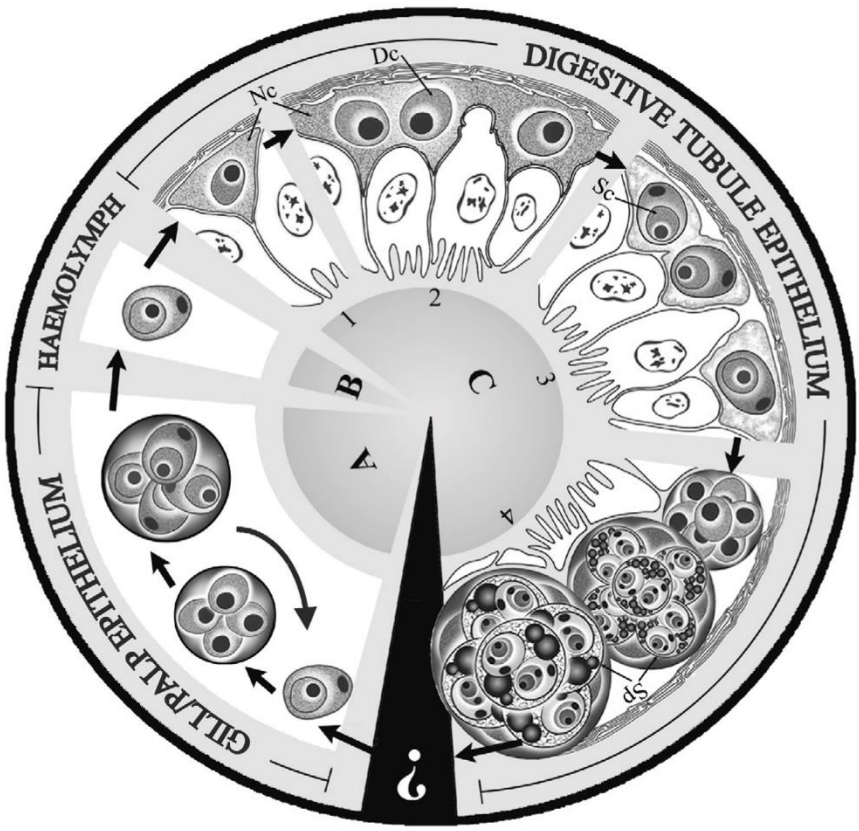

Figure 1-4: The life cycle of *Marteilia sydneyi* in Sydney rock oyster. The parasite from unknown source enters the gill/palp epithelium (A) then, the parasites mitigate through haemolymph (B) into the digestive tubule epithelium (B) where sporulation proceeds to forming mature sporonts (Sp). Reproduced with permission from Elsevier (Kleeman *et al.*, 2002).

QX disease infection often occur from January to April (Nell, 2007; Rubio *et al.*, 2013) with mortalities occurring from February to October (Wolf, 1979). The main signs of infection include extremely poor oyster condition with the gonads completely resorbed, a shrinking body (Wolf, 1979) and a change in the digestive gland from a dark to a pale yellowish colour (Figure 1-5) (DAWE, 2020). The colour change is a result of the development of QX spores in this tissue (Wolf, 1979). There is no difference in mortality rates between oysters of 1 year and 2-

3 years old (Wolf, 1979) with mortality rates of up to 97% after one season of exposure for both (Dove *et al.*, 2013a).

Figure 1-5: Disease symptom in a QX infected oyster. The digestive gland of a QX infected oyster has a pale yellowish colour (on left) while the digestive gland of a healthy oyster has a dark colour (on right). Reproduced according to conditions required by the Australian Government, Department of Agriculture, Water and the Environment (Department of Agricultre, Water and the Environment, 2020).

Given that gross signs of QX infection are not always specific, and a diagnostic presence of the parasite is needed, there are three methods that can be used to identify the parasite, including histology, tissue imprints and DNA based methods (Adlard & Wesche, 2005; Department of Agriculture, Water and the Environment, 2020; Kleeman & Adlard, 2000). The digestive gland is selected for the presence of the pathogen because it is the target organ of the parasite (see Figure 1-3 – The life cycle of *M. sydneyi* in SRO). In tissue imprints, a fresh sample of the digestive gland is taken, and excess water removed before imprinting onto a glass slide and staining with Hemacolor (Merck). The pathogen can then be easily observed under a light microscope (Figure 1-6) (Adlard & Wesche, 2005; da Silva & Villalba, 2004). For

histology, tissue sections are fixed with approximately 5 µm thick sections cut and placed onto a glass slide and stained using Hematoxylin and Eosin (H&E). The presence of the pathogen is easily observable under a light microscope (Adlard & Wesche, 2005; Department of Agriculture, Water and the Environment, 2020). Tissue imprints are quicker and cheaper than the histological technique (da Silva & Villalba, 2004). Tissue imprints and histology have lower sensitivity than DNA-based diagnostic methods, such as the polymerase chain reaction (PCR) (Adlard & Wesche, 2005; Kleeman & Adlard, 2000). For PCR detection of the QX pathogen, primers that target the ITS1 rDNA of *M. sydneyi* are used and amplify a 195-bp fragment which is sensitive enough to repeatedly amplify a DNA concentration equivalent to 0.01 sporont (Kleeman & Adlard, 2000).

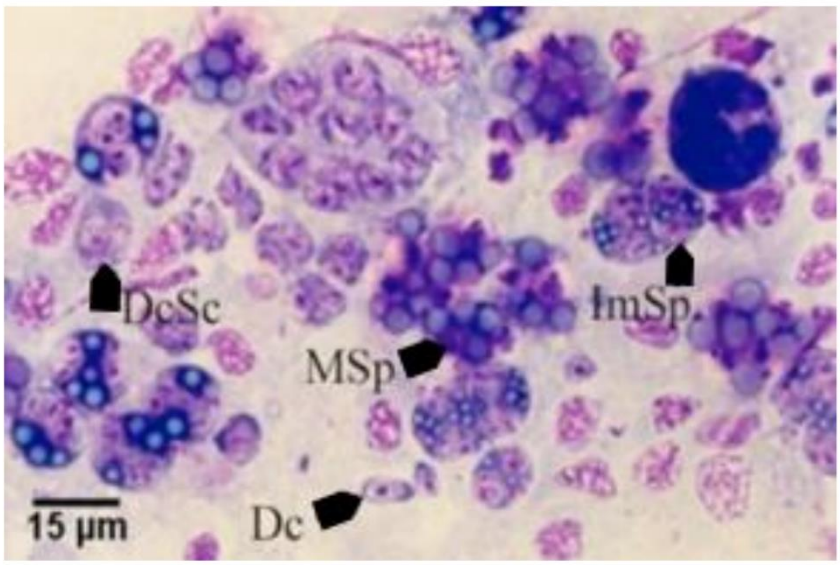

Figure 1-6: Tissue imprint of the digestive gland of SRO infected with *Marteilia sydneyi*. Various development stages of the parasite include daughter cells (Dc), daughter cells containing secondary cells (DcSc), immature sporonts (ImSp), and mature sporonts (MSp) showing in the tissue section. Reproduced with permission from the Australian Government, Department of Agriculture, Water and the Environment (Department of Agriculture, Water and the Environment, 2020).

It is notable that *M. sydneyi* can be present in SRO and not be associated with mortality (Adlard & Wesche, 2005) indicating that other factors, beyond the presence of the pathogen, are important for infection or progression of disease. As with other diseases, QX disease outbreaks are likely driven by the specific interactions between the oyster, the *M. sydneyi* parasite and the environment (Department of Agriculture, Water and the Environment, 2020; Green *et al.*, 2011; King *et al.*, 2019a; Raftos *et al.*, 2014) (Figure 1-7). There is evidence that QX outbreaks do not regularly occur in all oyster culture areas with some estuaries having never experienced this disease despite the presence of the pathogen (Adlard & Wesche, 2005; Department of Agriculture of Agriculture, Water and the Environment, 2020). QX disease outbreaks often occur after heavy rain resulting in reducing salinity and pH in the water column (Anderson *et al.*, 1994; Lester, 1986; Rubio *et al.*, 2013). Whilst there is no correlation between fluctuation in pH and QX disease (Anderson *et al.*, 1994; Wesche, 1995), there is a link with low salinity (Butt *et al.*, 2006; Newton *et al.*, 2004; Peters & Raftos, 2003). Low salinity has been shown to correlate with decreased phenoloxidase (PO) activity in SROs (Butt *et al.*, 2006), an enzyme in invertebrates involved in immune responses (Söderhäll & Cerenius, 1998). There is evidence that QX disease can be terminated when a significant decrease in water temperature occurs at a rate of approximately 3.7 ^{0}C per month (Rubio *et al.*, 2013), and no QX disease outbreak has been recorded with water temperature less than 21.5 ^{0}C (Rubio *et al.*, 2013). Additionally, there is a link between anthropogenic disturbance of the catchment including clearing land for agriculture and urban development and QX disease outbreaks (Department of Agriculture, Water and the Environment, 2020). Declining water quality delivered from highly modified catchments is known to affect immunity of the oysters which likely increases susceptibility to the QX pathogen (Diggles, 2013).

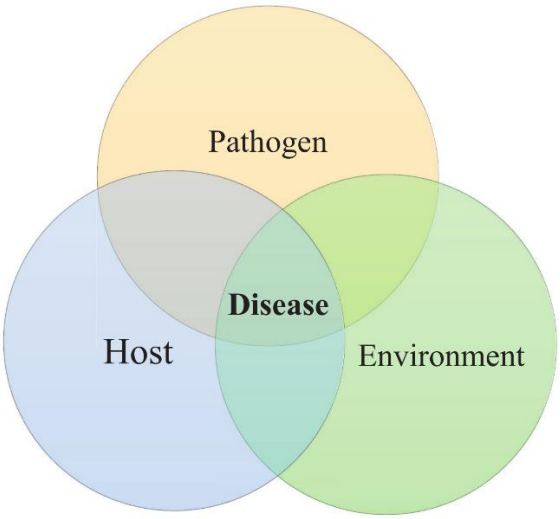

Figure 1-7: The relationship between host, pathogen, and environmental factors in disease occurrence. In terms of host factors, genetics is as an important variable in host susceptibility to disease (Hedrick, 1998). Oyster genetics also influences the microbiota (King *et al.*, 2019c; Wegner *et al.*, 2013) however, how the microbiota might influence disease susceptibility/resistance is largely unknown. With that said, it is known is that the host microbiota can enhance host immunity (Desriac *et al.*, 2014; Kamada *et al.*, 2013) and can host opportunistic pathogens (Cerf-Bensussan & Gaboriau-Routhiau, 2010).

1.1.5 Breeding for QX disease resistance

The government agency responsible for managing aquaculture activities in estuaries where SROs are cultivated – the New South Wales Department of Primary Industries (NSW DPI) - has led a selective breeding program since 1990 (Nell *et al.*, 2000). Initially, the breeding program aimed to increase growth rate and resistance to winter mortality (WM) with success leading to second generation SROs having a reduced time to market size (50g whole weight) of 3 months. Breeding strategies on reducing the impact of WM still require further investigation (Nell *et al.*, 2000). When QX disease occurred in Georges River in 1994 it killed

approximately 85% of the breeding population in this estuary which disrupted the breeding program (Nell *et al.*, 2000). In 1997, the program was modified to include breeding for QX disease resistance using mass selection methods. Second generation QX survivors were used as parents and exposed to QX disease in the George River with the best QX survivors selected as parents for the third generation of SROs (Nell *et al.*, 2000) and fourth generation (Dove *et al.*, 2013a; Dove *et al.*, 2013b). Studies have indicated WM resistance of third and fourth generations of SROs from the breeding program to have approximately 10% to 20% lower mortality, respectively, compared to controls (Dove *et al.*, 2013b; Nell & Perkins, 2006). Third generation selectively bred SROs for QX disease resistance had approximately 29% reduced mortality compared to controls (Nell & Perkins, 2006) and fourth generation reduced mortality from QX disease to 50% compared to controls (Dove *et al.*, 2013a; Dove *et al.*, 2013b). A recent study has confirmed these results with the breeding program greatly reducing SRO mortalities in the field, with some families having 85% survival through one cycle of QX disease (Dove *et al.*, 2020).

In terms of the mechanism for QX disease resistance, selectively bred oysters have higher phenoloxidase (PO) activity when compared to non-selected oysters (Butt & Raftos, 2008; Newton *et al.*, 2004). As mentioned above, PO is involved in oyster immunity (Aladaileh *et al.*, 2007a; Aladaileh *et al.*, 2007b; Aladaileh *et al.*, 2007c; Butt & Raftos, 2008) and is implicated in the synthesis of antimicrobial metabolites such as the pigment melanin which has been observed surrounding *M. sydneyi* in the hemolymph of SROs (Butt & Raftos, 2008). Melanin works by physically shielding and, preventing growth of pathogens and by producing highly reactive and toxic quinone intermediates (Cerenius & Söderhäll, 2004). Previous studies have observed that PO is inhibited in QX-affected disease estuaries with low salinities which may be partly responsible for driving QX disease outbreaks (Butt *et al.*, 2006; Peters & Raftos,

2003). Furthermore, oysters bred for QX disease resistance have significantly higher expression of an extracellular superoxide dismutase (ecSOD) and they have lower peroxiredoxin 6 (Prx6) expression than non-resistant oysters (Green *et al.*, 2009). Proteomic and mass spectrometry revealed six proteins correlated in the hemolymph of QX disease resistant oysters with two proteins homologous to superoxide dismutase proteins from other oyster species (Simonian *et al.*, 2009). Therefore, it is likely that SROs selectively bred for QX resistance generate the antiparasitic compound, hydrogen peroxide (H_2O_2), at a higher concentration than non-selectively bred SROs (Green *et al.*, 2009).

Despite the important role of oyster immunity in QX disease, the full mechanism(s) for QX disease resistance is likely unresolved. A recent study has shown that Pacific oysters bred for resistance to the viral pathogen OsHV-1 have different microbiotas (King *et al.*, 2019c) and that the microbiota composition may be associated with a differential outcome when challenged with OsHV-1 (Pathirana *et al.*, 2019).

1.1.6 Microbiota

1.1.6.1 The microbiota and host health

Microbiota is defined as the collection of microorganisms found in a defined environment (Marchesi & Ravel, 2015). In aquatic organisms, there are multiple relationships that may exist between the microbiota and a host that may affect physiological functions such as nutrient absorption, immune responses and disease susceptibility/resistance (Crosby *et al.*, 1990; Desriac *et al.*, 2014; Mueller & Sachs, 2015; Rawls *et al.*, 2004). Additionally, the microbiota may be a food source (Brown *et al.*, 1996; Prieur *et al.*, 1990), provide synthesised nutrients such as amino acids (Brown *et al.*, 1996), promote fatty acid absorption (Semova *et al.*, 2012) or enhance digestion (El-Shanshoury *et al.*, 1994; Erasmus *et al.*, 1997). The microbiota can

enhance the hosts' immunity by providing resistance from external pathogens (Desriac *et al.*, 2014; Kamada *et al.*, 2013) by competing for colonization sites (Reid *et al.*, 2001), competing for nutrients, stimulate the host immune system (Gatesoupe, 1999) or provide anti-pathogen activities such as production of inhibitory compounds (Offret *et al.*, 2019; Prado *et al.*, 2009). However, the microbiota can also have negative impacts on the host organism, for example, opportunistic pathogens such as *Vibrio* species are often members of a microbiota (Alsina & Blanch, 1994; Cerf-Bensussan & Gaboriau-Routhiau, 2010) that can cause mass mortalities in bivalves (Beaz-Hidalgo *et al.*, 2010). For the remainder of this section, the microbiota will be the focus since most research on oyster microbial communities have focussed on the bacterial component and, because this is what was investigated in this thesis.

1.1.6.2 The oyster microbiota

1.1.6.2.1 The composition of the oyster microbiota

Historically, many studies have isolated bacteria from bivalves using culture-based methods (Colwell & Liston, 1960). Using this method, the culturable bacterial community of Pacific oysters is dominated by bacteria from genera that include *Aeromonas, Alteromonas, Pseudomonas, Flavobacterium* and *Vibrio* (Colwell & Liston, 1960; Olafsen *et al.*, 1993; Pujalte *et al.*, 1999; Vasconcelos & Lee, 1972). However, the utility of these studies is limited because less than 0.001% bacteria can be cultured (Romero & Espejo, 2001). In recent years, most studies on the microbiota of oysters have used non-culturable methods such as 16S rRNA gene amplicon sequencing from metagenomic DNA providing substantially more resolved information on the relative abundance of operational taxonomic units (OTUs) within target tissues of oysters (Chauhan *et al.*, 2014; Dubé *et al.*, 2019; King *et al.*, 2019b; King *et al.*, 2019c; Lokmer *et al.*, 2016a; Pierce & Ward, 2019). These studies have demonstrated that the

bacterial communities of oysters are dominated by the *Proteobacteria* and *Tenericutes* phyla (Chauhan *et al.*, 2014; Dubé *et al.*, 2019; King *et al.*, 2012; Lokmer *et al.*, 2016a; Wegner *et al.*, 2013) and genera such as *Mycoplasma* (King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013), *Arcobacter* (Lokmer *et al.*, 2016a; Wegner *et al.*, 2013) and *Vibrio* (King *et al.*, 2019b; King *et al.*, 2019c). Bacteria of the *Mycoplasma* genus are consistently associated in high relative abundance with healthy oysters including Pacific oysters (*Crassostrea gigas*), Eastern oysters (*Crassostrea virginica*) and SRO (Green & Barnes, 2010; King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013) suggesting they may have a beneficial role. Alternatively, some taxa can be problematic such as those of the *Vibrio* genus. For example, *Vibrio* bacteria are significantly more abundant in Pacific oyster families that are sensitive to the viral pathogen OsHV-1 (King *et al.*, 2019b). OsHV-1 is known to inhibit the Pacific oyster immune system allowing *Vibrio* species to cause secondary infection (de Lorgeril *et al.*, 2018). As *Vibrio* bacteria are already part of the microbiota in these sensitive families, secondary infection is more likely to occur.

1.1.6.2.2 Factors that influence the oyster microbiota

Despite the extensive filter feeding nature of oysters, their microbiota structure is highly distinct from the microbiota in the surrounding seawater (Lokmer *et al.*, 2016a; Lokmer *et al.*, 2016b) indicating that the oyster microbiota is governed by fundamentally different processes than the surrounding water community. The microbiota of oysters can be influenced by multiple factors such as location, season, genetics and disease (see Table 1-1 for a summary of studies identifying factors affecting microbiota composition). The effect of spatial and temporal factors in the oyster microbiota is likely driven by changing environmental conditions (Lokmer *et al.*, 2016a; Pierce *et al.*, 2016) such as temperature (Levican *et al.*, 2014; Lokmer & Wegner, 2015; Lokmer *et al.*, 2016a; Motes *et al.*, 1998; Pierce *et al.*, 2016; Pujalte *et al.*,

1999) and salinity (del Refugio Castañeda Chávez *et al.*, 2005; Froelich *et al.*, 2016; Motes *et al.*, 1998). For example, under a set of controlled laboratory conditions, alpha diversity of the microbiota of Pacific oysters was significantly higher at a warmer temperature (22 ^{0}C) when compared to colder temperatures (8 ^{0}C) (Lokmer & Wegner, 2015). Additionally, some studies have shown that warm temperature positively correlates with the abundance of problematic bacteria such as *Vibrio* in Eastern oysters in the United States (Froelich *et al.*, 2016; Motes *et al.*, 1998) and *Arcobacter* in Pacific oysters collected from Spain (Levican et al., 2014) which likely explains (at least in part) the importance of warmer temperature in infectious outbreaks. Furthermore, other studies have indicated other environmental parameters such as salinity and total suspended solids as factors in the abundance of *Vibrio* species in Eastern oysters (del Refugio Castañeda Chávez *et al.*, 2005; Froelich *et al.*, 2016; Motes *et al.*, 1998).

Besides spatial and temporal factors, the oyster microbiota is variable between individuals (Wegner *et al.*, 2013) indicating host factors can also play a role. For example, Pacific oysters bred to be resistant to the viral OsHV-1 pathogen (King *et al.*, 2019c) show modified microbiota composition. Specifically, the oyster microbiota was significantly different between Pacific oyster families exhibiting different levels of susceptibility to OsHV-1 µvar disease with a significant increase in the abundance of potentially pathogenic bacteria such as *Vibrio* in the OsHV-1 sensitive families (King *et al.*, 2019c). Furthermore, the disease itself is a factor on oyster microbiota (Green & Barnes, 2010; King *et al.*, 2019b). The bacterial diversity significantly decreased in SROs associated with QX disease with the bacterial community being dominated by an OTU closely related to a *Rickettsiales*-like prokaryote (Green & Barnes, 2010). In Pacific oysters, summer mortality does not affect bacterial diversity. However, it significantly influences the microbiota composition with a significant increase in the abundance of *Vibrio* genus in the summer disease affected Pacific oysters (King *et al.*, 2019b).

Table 1-1: A survey of studies highlighting factors affecting oyster microbiota

Factor	Oyster species	Explanation	Reference
Location	*C. gigas*	The oyster microbiota significantly different between locations with *Oceanospirillaceae* family was identified as a main driver of the microbiota dissimilarity between locations.	(Lokmer *et al.*, 2016a)
Location	*C. virginica*	The oyster microbiota was significantly different between locations with *Mycoplasma, Planctomyctes, Shewanella and Chloroflexi* identified as main drivers of the microbiota dissimilarity between locations.	(King *et al.*, 2012)
Season	*Spondylus spinosus*	The oyster microbiota in summer season significantly differed from those in spring and winter with alpha diversity (Shannon index) in oysters sampled at a temperature above 30^0C being significantly higher when compared to oysters collected at a temperature below 30^0C. An increase in the relative abundance of the phylum *Spirochaetes* was observed in oysters sampled in summer.	(Roterman *et al.*, 2015)

Season	*Chama pacifica and C. savignyi*	The microbiota of oysters differed significantly between summer and winter. Species richness (Chao1) was significantly higher in summer compared to winter. The relative abundance of *Proteobacteria* was over-represented in summer compared to winter season.	(Zurel *et al.*, 2011)
Season	*C. virginica*	The microbiota significantly differed between colder months (<10ºC) and warmer months (>20ºC) with the microbiota more tightly grouped during colder months. *Vibrio* spp. was only observed at temperature above 20ºC.	(Pierce *et al.*, 2016)
Breeding	*C. gigas*	The microbiota was significantly different between families that were susceptible and resistant to the viral pathogen OsHV-1 with the relative abundance of *Photobacterium*, *Vibrio*, *Aliivibrio*, *Streptococcus,* and *Roseovarius* genera higher in susceptible families compared to resistant families.	(King *et al.*, 2019b)
Disease	*C. gigas*	The microbiota of summer mortality disease-affected oysters is significantly different when compared those to disease-unaffected oysters with members belonging to the *Vibrio* genus significantly more abundant in the disease-affected.	(King *et al.*, 2019b)
Disease	*S. glomerata*	Bacterial diversity significantly decreased in the SROs associated with QX disease with the bacterial community being dominated by an OTU closely related to a *Rickettsiales*-like prokaryote.	(Green & Barnes, 2010)

1.1.6.2.3 Core microbiota

Because of the diversity, complexity and high dynamic nature of microbiota, it can be quite challenging to identify key taxa that may have an important role in a host. To overcome this, some studies have focused on the core microbiota in marine organisms such as sponges (Schmitt *et al.*, 2012) and coral (Ainsworth *et al.*, 2015; Lawson *et al.*, 2018). A core microbiota is defined as the members shared among microbiotas from similar habitats (Shade & Handelsman, 2012). Based on this definition, there are several methods that can be used for core analysis such as a core based on shared OTU occurrence, shared abundance, shared OTU lineages, OTU shared over time and across communities (Shade & Handelsman, 2012). Among these methods, a core microbiota based on shared OTU occurrence has been applied in many studies (Hernandez-Agreda *et al.*, 2018; Huse *et al.*, 2012; King *et al.*, 2012; King *et al.*, 2019c; Lawson *et al.*, 2018; Schmitt *et al.*, 2012). However, the definition of core OTU within the community is not unified. For example, a core microbiota has been defined as the OTU present in at least 50% of samples (Hernandez-Agreda *et al.*, 2016), or at least 70% (Schmitt *et al.*, 2012) or 100% (Lawson *et al.*, 2018). Therefore, depending on the ecological question, an appropriate definition of core microbiota is applied to identify the stable and consistent members across complex microbial assemblages (Shade & Handelsman, 2012).

Only a few studies have characterised the core microbiota in oysters. A core microbiota of the Eastern oyster was defined based on phylotypes with OTUs present in 100% of stomach and gut samples (King *et al.*, 2012). The result indicated that 5 OTUs in 3 phyla were identified as the core stomach microbiota, while the core gut microbiota comprised of 44 OTUs from 12 phyla (King *et al.*, 2012). In a study looking at the influence of genetics on the microbiota of 35 Pacific oyster families sampled at the same time from the same environment (King *et al.*, 2019c), the core microbiota was investigated for OTUs present in at least all but one family

replicate (King *et al.*, 2019c). There was no universal core microbiota across all of the 35 Pacific oyster families however, the *Winogradskyella* genus and the *Bradyrhizobiaceae* family were identified as core microbiotas from the highest OsHV-1 resistant group (King *et al.*, 2019c). Although some core taxa were identified, the functions of these members in oysters remain unknown (King *et al.*, 2012; King *et al.*, 2019c).

1.1.6.3 The oyster microbiota and disease

The oyster microbiota is comprised of unique bacterial communities (King *et al.*, 2012; Lokmer *et al.*, 2016b) and can have positive or negative effects on the oyster health (Lokmer *et al.*, 2016b). For example, *Pseudoalteromonas* spp. can produce antibacterial compounds which are known to be active against a variety of target organisms (Defer *et al.*, 2013; Desriac *et al.*, 2014). In contrast, many species are potential oyster (opportunistic) pathogens such as those from the *Arcobacter* (de Lorgeril et al., 2018), *Vibrio* (de Lorgeril *et al.*, 2018; King *et al.*, 2019b) and *Roseobacter* (Boettcher *et al.*, 2000) genera, and, taxa from the Spirochaete phylum (Matsuyama *et al.*, 2017). Particularly important are several *Vibrio* species that have been shown to cause mortality in oysters (Table 1-2) (Paillard *et al.*, 2004).

In Pacific oysters, the microbiota is emerging as a factor in disease dynamics (King *et al.*, 2019b; Petton *et al.*, 2015; Wendling *et al.*, 2014). While specific *Vibrio* appear necessary for summer mortality, non-virulent *Vibrio* may also play a role by dramatically increasing the virulence of pathogenic *Vibrio* strains when co-provided at low doses (Petton *et al.*, 2015). Additionally, antibiotic treatment of oysters prior to disease challenge substantially reduced mortality (Petton *et al.*, 2015) indicating that the indigenous microbiota might play an important role in Pacific oyster diseases.

In regard to SROs, the literature shows only one study exploring the bacterial community and these were SROs associated with QX disease. In this study, the digestive gland microbiota of *M. sydneyi* infected SROs was dominated by an OTU assigned to *Rickettsiale*-like prokaryotes (Green & Barnes, 2010). Whether changes in the SRO microbiota is a result of QX disease infection or destabilisation of the SRO microbiota prior to infection is unknown (Green & Barnes, 2010) so it remains unclear whether the *Rickettsiale*-like OTU has any role at all.

Table 1-2: Pathogenic *Vibrio* species to oysters

Bacteria species	**Oyster species**	**Growth stage**	**References**
Vibrio sp.	*C. gigas*	Larvae	(Estes *et al.*, 2004; Garland *et al.*, 1983)
	C. gigas	Juveniles	(Gay *et al.*, 2004)
	C. virginica	Larvae	(Elston & Leibovitz, 1980)
V. tubiashii	*C. gigas*	Larvae	(Hasegawa *et al.*, 2008)
V. splendidus	*C. gigas*	Juveniles	(Lacoste *et al.*, 2001)
V. aestuarianus	*C. gigas*	Adult	(Garnier *et al.*, 2007)
V. anguillarum	*C. virginica*	Larvae	(Tubiash, 1975)

1.2 Research aims

In other oyster species, the microbiota is influenced by both environmental and host factors such as location, season, genetics and disease state. However, there is a paucity of studies examining the factors that influence the microbiota assemblage of the SRO. It is hypothesized that the microbiota of SROs is different between geographic locations, seasons and genetics, and that there are key taxa indicative of healthy and QX infected SROs. To determine whether these hypotheses are true, this thesis has set out to address three aims, with the overall objective

to characterise the SRO microbiota and, investigate its relationship to QX disease. The specific aims of the research are:

1. To explore the influence of geographic location, season and genetics (breeding for QX disease resistance) on the SRO microbiota (Chapter 2).
2. To characterise the microbiotas of SRO prior to and during a QX disease event (Chapter 3).
3. To identify key indicator taxa of healthy and QX-infected oysters (Chapter 4).

Chapter 2 The Sydney rock oyster microbiota is influenced by location, season and genetics

Chapter two – Declaration

I declare that the below publication meets the below requirements for inclusion as a chapter in this thesis.

- I have contributed more than 50% for the below publication.
- The below publication has been peer reviewed.
- The below publication has been formally published, and is formatted to adhere to the specific formatting requirements of Aquaculture.
- Permission is not required by the publisher for inclusion of this publication in this thesis for non-commercial purposes.

Nguyen, V. K., King, W. L., Siboni, N., Mahbub, K. R., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). The Sydney rock oyster microbiota is influenced by location, season and genetics. *Aquaculture*, 735472.

doi:https://doi.org/10.1016/j.aquaculture.2020.735472

Publication status: Published

The full published version of the manuscript can be found in the appendix

Date: 10th February 2021

Candidate's signature: Production Note: Signature removed prior to publication.

The Sydney rock oyster microbiota is influenced by location, season and genetics

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2.1 Abstract

Queensland unknown (QX) disease is a significant cause of economic loss for the Sydney rock oyster (SRO; *Saccostrea glomerata*) aquaculture industry. Evidence is emerging that QX disease is multi-factorial in nature, with a number of environmental and host factors contributing to disease dynamics. Efforts to mitigate the impacts of QX disease are primarily focused on breeding for disease resistance however, the mechanisms that drive disease resistance are poorly understood. One potential factor influencing disease resistance is the microbiota. To determine the influence of location, season and disease resistance on the SRO microbiota, we used 16S rRNA gene (V1 – V3 region) amplicon sequencing. The microbiota of six SRO families with two categorised as QX-resistant and four as QX-susceptible, deployed to two different locations (Port Stephens and Wallis Lake, NSW, Australia) and over two seasons (Austral summer and winter), were characterised. As expected, the SRO microbiota was distinct to the microbial community found in seawater. Further, the SRO microbiota was significantly influenced by location and season, with operational taxonomic units (OTUs) assigned to the *Candidatus Hepatoplasma* and *Endozoicomonas* genera identified as significant drivers of microbiota dissimilarity between locations and seasons. Disease resistance also significantly influenced the SRO microbiota but only at the winter time point which is before the typical QX disease period. Overall, OTUs assigned to the *Mycoplasma*, *Borrelia* and *Endozoicomonas* genera were over-represented in QX-resistant SRO microbiota, whereas members of the *Pseudoalteromonas*, *Vibrio*, and *Candidatus Hepatoplasma* genera were over-represented in QX-sensitive microbiota. These findings confirm the influencing role of location and season on the microbiota structure as evidenced in other molluscan species, but also provide preliminary evidence that the microbiota assemblage before the QX disease period may be important for resistance to disease and may provide new avenues for managing SRO aquaculture in the future.

Keywords: Microbiota, Sydney rock oyster, QX disease, 16S rRNA gene, disease resistance

2.2 Introduction

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is native to Australia, where it is one of the most intensively cultivated oyster species (O'Connor & Dove, 2009; Schrobback *et al.*, 2014). However, since the mid-1970's production of this species has been impacted by Queensland unknown (QX) disease, which can recurrently cause up to 90% mortality in affected estuaries (Department of Primary Industries, 2016; Nell, 2007; O'Connor & Dove, 2009; Peters & Raftos, 2003; Schrobback *et al.*, 2014). The aetiological agent for QX disease is a spore-forming protozoan parasite called *Marteilia sydneyi.* This parasite has an infection cycle that typically enters through the palps and gills in summer and ends in the oyster digestion gland, impacting nutrient uptake and ultimately causing starvation and death through autumn and into winter (Kleeman *et al.*, 2002; Nell, 2007).

To mitigate the impacts of QX disease, the New South Wales Department of Primary Industries (NSW DPI) has led a selective breeding program using both mass selection methods and family based breeding that has greatly reduced SRO mortalities, with some families showing 85% survival through one cycle of disease (Dove *et al.*, 2020). There is evidence that increased levels of resistance in some families may be linked to higher activity of phenoloxidase, an enzyme thought to be involved in oyster defence mechanisms (Newton *et al.*, 2004), yet the full mechanism(s) for resistance remain unresolved.

The oyster microbiota is emerging as a factor in disease dynamics (King *et al.*, 2019a) and is an unexplored factor in SRO QX disease resistance. The potential protective role of the mollusc microbiota has been characterised previously, with some microbial members providing anti-pathogen activities (Offret *et al.*, 2019; Prado *et al.*, 2009). In other studies, the microbiota

appears to contribute to disease dynamics, for the Pacific oyster (*Crassostrea gigas*) it has been demonstrated that summer mortality in France is due to a progressive replacement of non-virulent commensal vibrios with pathogenic vibrios indicating that microbiota dysbiosis precedes mortality (Lemire *et al.*, 2015). Similarly, Pacific oyster mortality syndrome is polymicrobial in nature with a recent study showing that the viral Ostreid Herpesvirus 1 (OsHV-1) suppresses Pacific oyster immunity, allowing opportunistic bacterial pathogens such as *Vibrio* species to thrive (de Lorgeril *et al.*, 2018). Interestingly, the microbiota of Pacific oyster families bred for resistance to OsHV-1 were significantly different to their disease-susceptible counterparts and had a significantly reduced abundance of *Vibrio* species (King *et al.*, 2019c). In SROs, only one study has investigated the QX disease-affected microbiota by comparing the digestive gland of QX-infected and uninfected oysters (Green & Barnes, 2010). In QX-infected oysters, bacterial diversity was substantially reduced, with the microbiota dominated by a *Rickettsiales*-like operational taxonomic unit (OTU).

A first step in understanding the role of a microbiota in disease dynamics is characterising its composition and determining the factors that shape its structure. In previous studies in other oyster species, the oyster microbiota has been shown to be influenced by both environmental and host factors including location, temperature, infection state, season, genetics, life stage and resistance to disease (Green & Barnes, 2010; King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2019c; Lokmer & Wegner, 2015; Lokmer *et al.*, 2016a). However, there is a paucity of studies examining the factors that influence the SRO microbiota assemblage. Therefore, to characterise the influence of location, season and disease-resistance (genetics) on the SRO microbiota, six SRO families with varying degrees of resistance to QX disease were deployed into two locations and sampled in the Austral summer and winter. Understanding the mechanism(s) that

drive disease-resistance, including the potential contribution of the microbiota to disease, are imperative for the successful and sustainable management of SRO aquaculture.

2.3 Materials and methods

2.3.1 Experimental design and sampling

Fourty-four different *Saccostrea glomerata* families from the 2015 year class were deployed in the Port Stephens (32°43'12.81"S 152°03'40.52"E) and Wallis Lake (32°11'21.3"S 152°29'09.7"E) estuaries in NSW, Australia. Wallis Lake is a wave-dominated barrier estuary whereas Port Stephens is a tide-dominated drowned valley estuary (Roy *et al.*, 2001). These estuaries are approximately 70 km apart and are not affected by QX disease. These sites were selected to remove the influence of infection- or disease-state on the microbiota. For this study, six families from the 2015 class were selected according to their predicted level of resistance to QX disease. Estimated Breeding Values (EBVs), which provide an estimation of how well families will perform for a particular trait and the likelihood of passing those traits to their progeny, were used to select the 6 families employed here. Subsequent exposure of these families to QX disease at Lime Kiln Bar in the Georges river (33°59'19"S 151°03'21"E) demonstrated that four of the families exhibited ≤50% survival (characterised as QX-susceptible), while the other two families displayed >50% survival (QX-resistant; Table 2-1). Five oysters per family were collected from each site in the 2017 Austral summer (January) and Austral winter (June), four and nine months after deployment respectively (120 oyster samples in total). Oysters were randomly collected by farmers from cultivation trays, placed into labelled plastic bags, transported to the laboratory on ice (3 - 4 hours) and stored whole in their shell at -80^{0}C for later processing. Because oyster leases could only be accessed by boat, seawater samples were collected from jetties (piers) approximately 800 metres away from the oyster leases. The Jetties in which the water were samples have a few meters of water beneath it ensuring no sediment was suspended from the bottom during the water collection. Ten litres of surface seawater samples were collected and kept on ice during transport to the laboratory.

Triplicate seawater samples of 2000 mL for each sampling time were filtered with Durapore Membrane Filters (0.22 µm pore size) for subsequent microbiota analyses, respectively. All filtered samples were frozen in liquid nitrogen upon collection in sterile 5 mL cryotubes and kept at -80^{0}C prior to analysis.

Table 2-1: 2015 year class Sydney rock oyster average family survival (n = 3, ± SD) following exposure to QX disease at Lime Kiln Bar, Georges river. Oysters were deployed to Lime Kiln Bar on 12 December 2016 and oyster survival was counted on 20 September 2017.

Family line	QX disease resistance level	Average survival (%)*
F25	Resistant	59.67 ± 0.58
F22	Resistant	55.33 ± 3.06
F18	Susceptible	19.67 ± 3.79
F03	Susceptible	3.33 ± 2.31
F32	Susceptible	2.67 ± 3.06
F37	Susceptible	0.67 ± 1.15

2.3.2 Measurement of environmental parameters, nutrients and chlorophyll a in seawater

Environmental parameters (temperature, oxygen, pH, and conductivity) were measured at jetties adjacent to the oyster leases using a WTW multiprobe meter (Multi 3430, Germany) at the time of oyster sample collections. For nutrient analysis, 50 mL triplicate seawater samples were syringe filtered through a 0.45 µm filter into 50 mL sterile falcon tubes, transported to the laboratory on ice, and frozen at -20^{0}C. Nutrient analysis (nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) and Phosphate (PO_4^{3-})) was performed by Envirolab Services Pty Ltd (Sydney,

New South Wales, Australia). From the 10 L of seawater collected above, triplicate 200 mL aliquots were filtered through glass microfiber filters (0.7 µm pore size) and stored at -20^{0}C for subsequent chlorophyll-a analyses. Chlorophyll a was analysed based on a Spectrophotometric method described previously (Ritchie, 2006).

2.3.3 DNA extractions and 16S rRNA gene amplicon sequencing

DNA extractions commenced only after the last sample had been collected and frozen. Samples were randomly thawed in batches of 20 and all samples were processes using a single DNA extraction kit. Thawed oysters 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 Petri dish. Approximately 25-50 mg of adductor muscle tissue was then excised using a sterile scalpel blade and placed into a 1.5 mL Eppendorf tube for subsequent DNA extraction using the Qiagen DNeasy Blood and Tissue DNA extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. Haemolymph is often used to study the oyster microbiota (Lokmer *et al.*, 2016a; Lokmer *et al.*, 2016b) but can be difficult to extract from small oysters and is not possible to extract once oysters have been frozen. To minimise variation, we decided to freeze oysters so they could be later processed together. Therefore, the adductor muscle was selected for microbiota analysis as it contains haemolymph sinuses thus allowing us to easily sample the haemolymph. This approach has been successfully used before (King *et al.*, 2019b; King *et al.*, 2019c). The instruments used to process the oysters, including the shucking knife, were cleaned, soaked in 1:15 bleach solution for 15 min and then rinsed with sterile Milli-Q water prior to use and between samples. DNA from filtered seawater samples were extracted using the PowerWater DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocol.

The V1–V3 region of the 16S rRNA gene was amplified by PCR using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′- GWATTACCGCGGCKGCTG-3′) primer pair (Lane, 1991; Turner *et al.*, 1999). The PCR cycling conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s and a final extension of 72°C for 10 min. Amplicons were sequenced using the Illumina MiSeq platform (2 × 300 bp) 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 (SRP234946) under Bioproject number PRJNA593911.

2.3.4 Bioinformatics analyses

Demultiplexed paired-end reads were combined using FLASH (Magoč & Salzberg, 2011) and trimmed using Mothur (Schloss *et al.*, 2009) (Parameters: maxhomop = 5, maxambig = 0, minlength = 471, maxlength = 501). Fragments were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and chimeric and singleton sequences were identified and removed using VSEARCH (Rognes *et al.*, 2016). Taxonomic assignment of OTUs were performed in QIIME version 1.9.1 (Caporaso *et al.*, 2010) using the UCLUST algorithm (Edgar, 2010) against the SILVA v128 dataset (Quast *et al.*, 2013). Mitochondrial and chloroplast data were filtered out of the dataset and then were rarefied to the same depth to remove the effect of sampling effort upon analysis. For beta diversity, the relative abundance of OTUs was calculated and all OTUs with a relative abundance below 0.1% were filtered from the dataset. Alpha diversity indices, including species richness (Chao1), species evenness (Simpson) and species diversity (Shannon index) were calculated using QIIME (Caporaso *et al.*, 2010).

2.3.5 Statistical analyses

Alpha diversity metrics were compared between groups using a Kruskal-Wallis test. All beta diversity analyses were performed with a Bray-Curtis dissimilarity index. To easily visualise how samples related to one another and observe distance matrices between groups, non-metric multidimensional scaling analysis (nMDS) with three dimensions (3D) was used. Patterns elucidated by the 3D nMDS were statistically tested using a permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations using transformed (square root(x)) data. To identify the OTUs driving the difference between the microbial assemblage at different locations or time points, SIMPER analysis was used. All alpha and beta diversity comparisons were performed in the PAST statistical environment (Hammer *et al.*, 2001). To determine whether the relative abundances of OTUs were significantly different between oyster groups with differing QX-resistance, Welch's T-Test was performed using the STAMP (Statistical Analysis of Metagenomic Profiles) software package version 2.1.3 (Parks *et al.*, 2014). A relative abundance of OTUs file was used as input data with the STAMP software along with a metadata file containing location, sampling time and QX-resistance group information. Welch's T-Test for two groups with a statistical cut-off of p-value >0.05 was used. Extended bar error bar plots with corrected p-values were produced using STAMP (Parks *et al.*, 2014) to visualise a significant difference in the relative abundance of OTUs between the QX-sensitive and resistant groups at a single location in each sampling time.

2.4 Results

Following amplicon sequencing of the 132 samples (oysters and seawater), data were rarefied to 7,178 reads retaining a total of 753,690 reads from 105 samples (Supplementary Table 2-1). After data filtering, a total of 1,889 OTUs were observed across the entire dataset. Of these, 1,619 and 190 OTUs were unique to the oyster and seawater microbiota respectively, with only 80 OTUs found in both the oyster and seawater samples.

2.4.1 The SRO microbiota is distinct from the seawater microbiota

Across the entire dataset, species richness, evenness and diversity were higher in seawater samples relative to the SRO adductor muscle microbiota (Figure 2-1 and Supplementary Table 2-2). When grouping all SRO or seawater samples, an 3D nMDS analysis revealed that the composition of the SRO and seawater microbiota were distinct from one another (Supplementary Figure 2-1), with these differences confirmed as significantly different by PERMANOVA ($F = 13.54$, $p = 0.0001$). SIMPER analysis revealed a 99.1% dissimilarity between the SRO and seawater microbiota, with *Candidatus Hepatoplasma* genus (OTU 14887) and *Endozoicomonas* genus (OTU 3829) over-represented in SRO microbiota and driving 5.7% and 2.9% of the difference respectively (Figure 2-2 and Supplementary Table 2-3). In seawater, the *Candidatus Actinomarina* genus (OTU 22961) and NS5 marine group genus (OTU 5409) were over-represented, driving 4.2% and 3.6% of the difference respectively (Figure 2-2 and Supplementary Table 2-3).

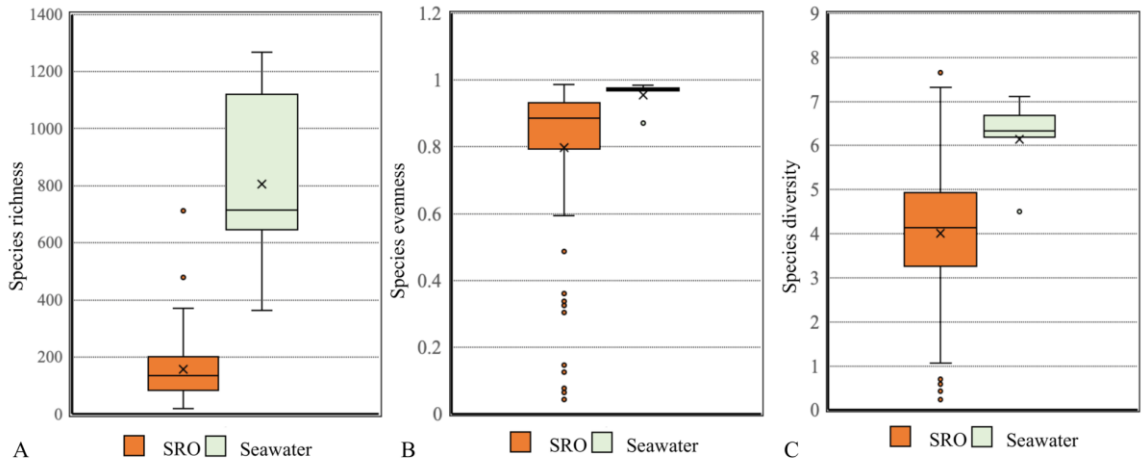


Figure 2-1: Box and whisker plot of species richness (Chao1) (A), evenness (Simpson) (B) and diversity (Shannon index) (C) for SRO and seawater microbiota. The x in the box plot is the mean of the dataset.

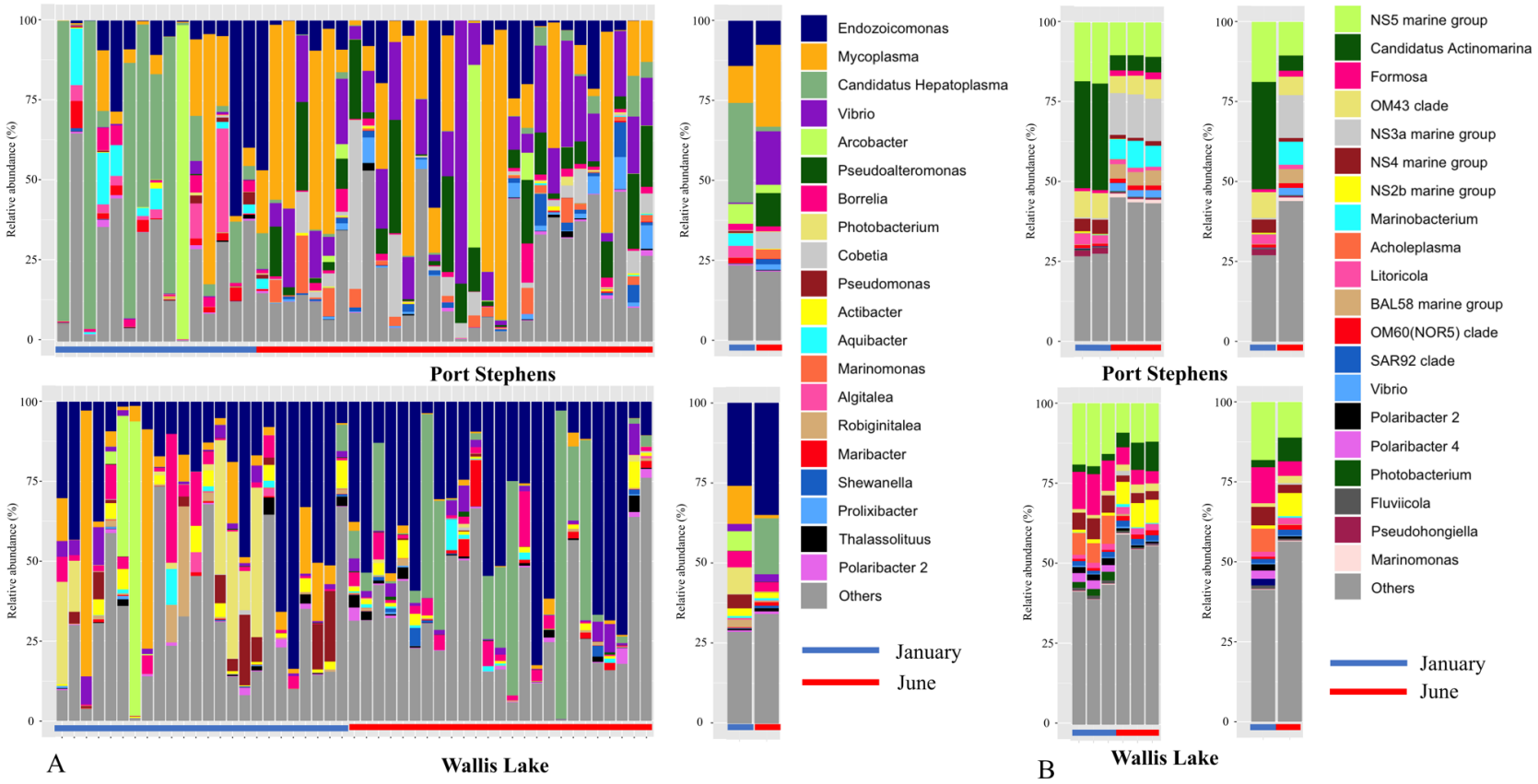


Figure 2-2: Microbiota composition of SRO (A) and seawater samples (B) in Port Stephens (upper panels) and Wallis Lake (lower panels) showing the top 20 dominant and remaining taxa in January (underlined by blue bar) and June (underlined by red bar). The right bars in each panel show the mean abundance of each taxon within each group. Data is summarised at the genus level.

2.4.2 Location is a factor shaping the SRO microbiota

Overall, Port Stephens had higher temperatures, pH and chlorophyll a at each time point, whereas Wallis Lake had higher levels of dissolved oxygen relative to Port Stephens. A rainfall event occurred during the June (winter) sampling at Port Stephens which likely explains the decrease in conductivity and increase in nutrients during this time point (Table 2-2).

When the total SRO microbiota deployed in Port Stephens and Wallis Lake were compared, species richness and diversity were statistically higher in Wallis Lake ($p = 0.029$ and $p = 0.007$ respectively, Supplementary Figure 2-2A and Supplementary Table 2-4). However, no statistical difference in alpha indices was observed when SRO microbiota from Port Stephens and Wallis Lake were independently compared in January and June (Supplementary Figure 2-2B and Supplementary Table 2-4).

Despite some overlap, a 3D nMDS plot showed that SRO microbiota clustered according to location (Figure 2-3A) and were significantly different according to site (PERMANOVA, F = 8.955, $p = 0.0001$). This effect of location was also evident within each season in January (Figure 3B and 3C; PERMANOVA, F = 5.117, $p = 0.0001$) and June (PERMANOVA, F = 11.81, $p = 0.0001$). Across the entire dataset, the SRO microbiota at Port Stephens and Wallis Lake were 90.5% dissimilar to one another. Similarly, in January and June, the SRO microbiota from the two sites were 90.3% and 91.9% dissimilar respectively. Interestingly, the main dissimilarity contributor, *Candidatus Hepatoplasma* genus (OTU 14887), was over-represented at Port Stephens in January contributing 17.7% to the dissimilarity between microbiota, however, was over-represented at Wallis Lake in June contributing 9.6% of the microbiota dissimilarity (Supplementary Table 2-5). Additionally, a member of the

Endozoicomonas genus (OTU 1831) was over-represented in Wallis Lake in both January and June contributing 3.0% and 6.4% respectively.

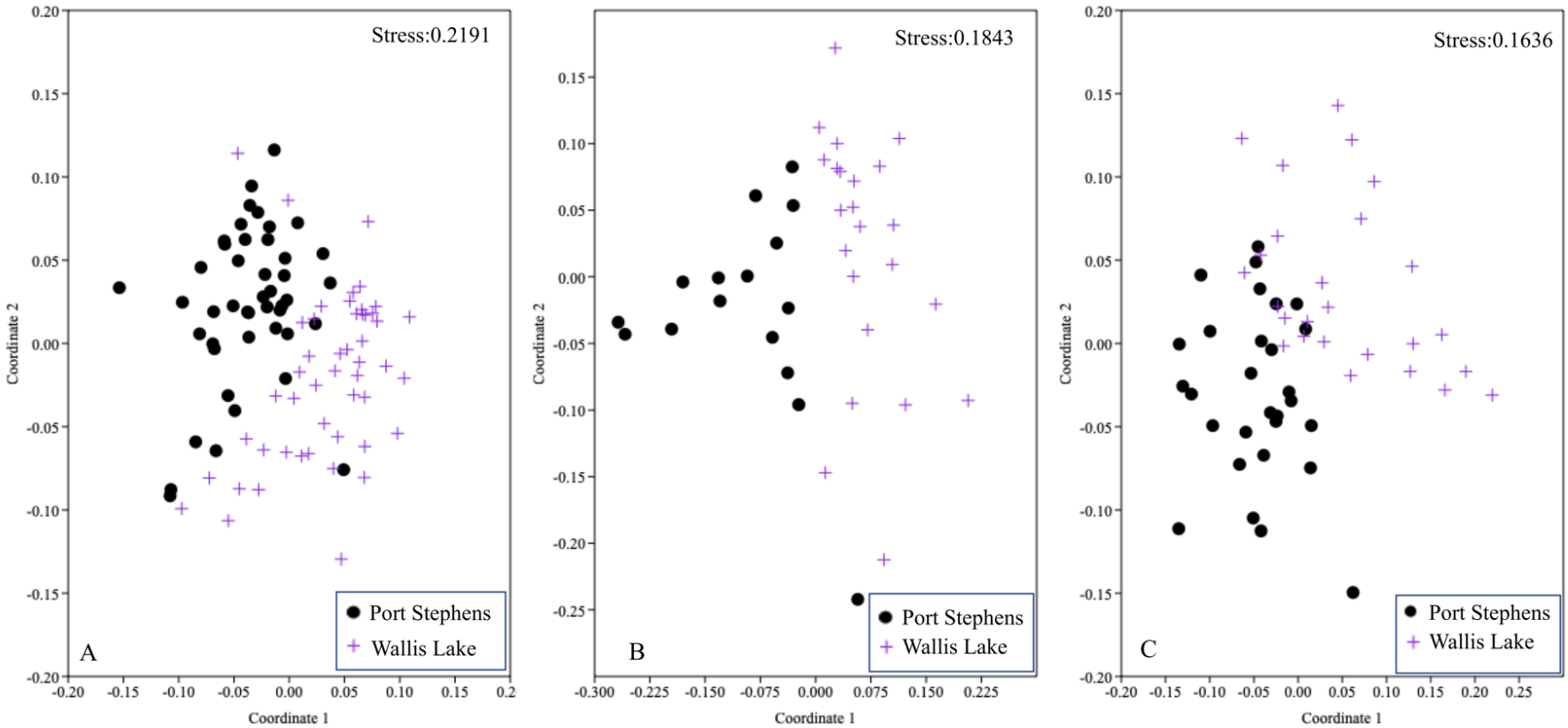


Figure 2-3: 3D nMDS plots based on Bray-Curtis dissimilarity for the comparison of the SRO microbiota of 94 oysters sampled across two time points (A) and 39 oysters sampled in January (B) and 55 oysters sampled in June (C) show separation according to location. Axes 1 and 2 are plotted.

Table 2-2: Environmental parameters in Port Stephens and Wallis Lake at time of sampling.

Time	Temperature (^{0}C)	pH	DO (mg/L)	Conductivity (µS/cm)	NO_3 (mg/L)	NO_2^- (mg/L)	NH_3 (mg/L)	PO_4^{3-} (mg/L)	Chlorophyll a (µg/ml)	Rainfall*
Port Stephens										
January	27.8	8.0	8.18	53.3	<0.005	0.004 ± 0.0	0.012 ± 0.003	0.014 ± 0.003	11.41 ± 1.48	Rainfall 2 days before sampling (0.4 mm). Monthly total rainfall was 69.9mm
June	24	8.3	8.88	27.6	0.047 ± 0.01	<0.005	0.038 ± 0.001	<0.005	23.03 ± 3.13	Rainfall over 6 days including during sampling (average 23.65 mm/day). Monthly total rainfall was 315.1mm
Wallis Lake										
January	24	7.2	9.5	53.9	<0.005	0.004 ± 0.0	0.013 ± 0.004	0.007 ± 0.001	9.05 ± 0.62	Rainfall event 2 days before sampling (2.0 mm). Monthly total rainfall was 89.2mm
June	18.3	8.2	9.07	53.6	0.014 ± 0.014	<0.005	0.018 ± 0.001	<0.005	9.52 ± 0.57	Rainfall over 3 days before sampling (average 5.6 mm/day). Monthly total rainfall was 188.1mm

*Data obtained from (Bureau of Meteorology, 2019)

2.4.3 Season is a factor shaping the SRO microbiota

We next examined whether seasonality influenced the SRO microbiota within a given location. There were no statistical differences in alpha diversity in either Port Stephens or Wallis Lake (Supplementary Figure 2-2C and Supplementary Table 2-4). However, 3D nMDS plots revealed the SRO microbiota at both sites tended to cluster according to sampling time (Figure 2-4). This seasonal variability was more pronounced in Port Stephens (PERMANOVA, F = 10.42, p = 0.0001) than Wallis Lake (PERMANOVA, F = 3.451, p = 0.0001). At Wallis Lake, the SRO microbiota was 86.5% dissimilar with OTUs assigned as members of the *Endozoicomonas* genus (OTU 1831) and the *Candidatus Hepatoplasma* genus (OTU 14887) over-represented in January and June respectively, contributing 8.1% and 10.4% to the microbiota dissimilarity (Supplementary Table 2-6). At Port Stephens, there was 92.7% dissimilarity in SRO microbiota composition between seasons, with an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) over-represented in January and contributing 16.8% to the dissimilarity. In June, OTUs assigned as *Vibrio* (OTU 2), *Mycoplasma* (OTU 14900) and *Pseudoalteromonas* (OTU 8917) were over-represented, contributing 6.6%, 5.6% and 5.0% to the dissimilarity between seasons respectively (Supplementary Table 2-6).

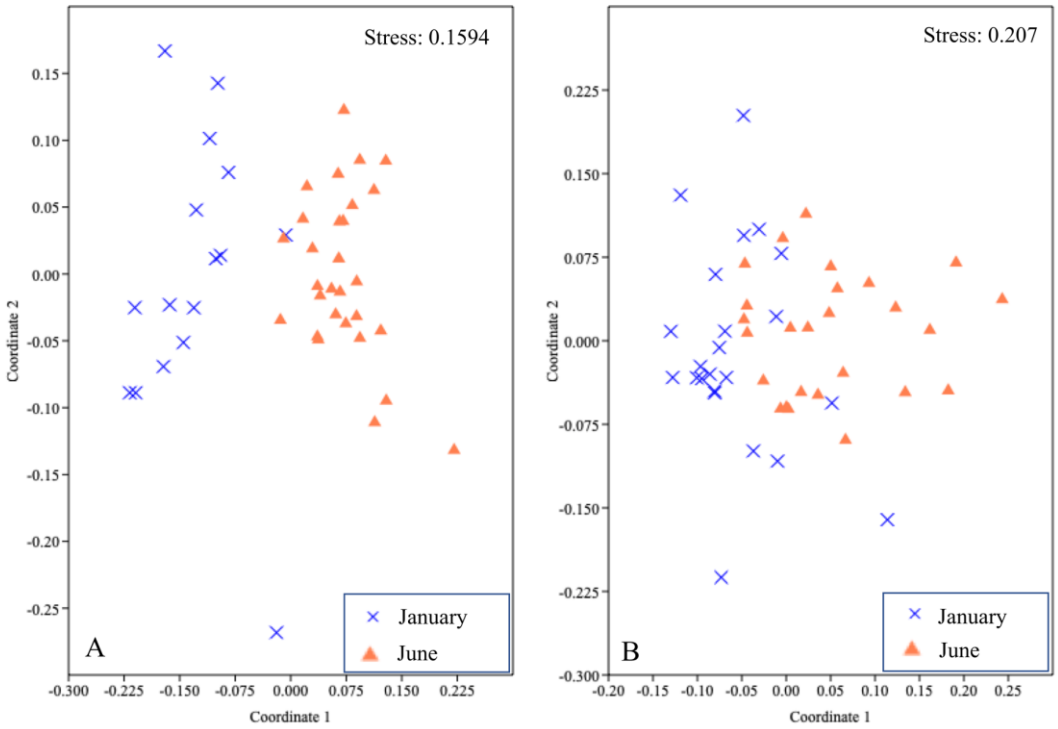


Figure 2-4: 3D nMDS plots based on Bray-Curtis dissimilarity for the comparison of the SRO microbiota of 45 oysters sampled in Port Stephens (A) and 49 oysters sampled in Wallis Lake (B) separate according to time of sampling. Axes 1 and 2 are plotted.

2.4.4 QX-resistance is a factor shaping the SRO microbiota

Across times and sites, we analysed differences in the oyster microbiota between SROs with different levels of resistance to QX disease. Families were grouped as QX-sensitive if survival was ≤50% and QX-resistant if displayed >50% survival (Table 2-1). Species richness was higher in the QX-sensitive group at Port Stephens in January (Average: 74 ± 3.26 vs 143.38 ± 77.87, $p = 0.039$; Supplementary Table 2-7). No other significant differences in alpha diversity indices were observed between the QX groups in each location at each time point (Supplementary Table 2-7).

PERMANOVA showed statistically significant differences in the microbiota structure of different QX-resistance groups only in June at both locations (Table 2-3).

At Port Stephens in June, SIMPER analysis revealed a 75.7% dissimilarity between the QX-sensitive and QX-resistant groups with two OTUs (OTU 12669 and OTU 14900) from the *Mycoplasma* genus over-represented in the QX-resistant group and contributing 9.6% and 9.2% to the microbiota dissimilarity. OTUs belonging to the *Pseudoalteromonas* (OTU 8917) and *Vibrio* (OTU 2) genera were over-represented in the QX-sensitive group contributing 6.4% and 6.1% to the microbiota dissimilarity (Supplementary Table 2-8), while another OTU assigned to the *Vibrio* genus (OTU 1) was over-represented in the QX-resistant microbiota contributing 5.6% dissimilarity (Supplementary Table 2-8). Additionally, two *Mycoplasma* OTUs (OTU 12669 and OTU 14900) were over-represented in the QX-resistant group, contributing 9.6% and 9.2% to the microbiota dissimilarity. At Wallis Lake in June, SIMPER revealed 77.9% microbiota dissimilarity between the QX groups. A member assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) was over-represented in the QX-sensitive group and contributed 15.86% of the microbiota dissimilarity, whereas 5 OTUs, all assigned to the *Endozoicomonas* genus (OTUs 1831, 3829, 6283, 3483 and 4530), were over-represented in the QX-resistant microbiota.

Table 2-3: PERMANOVA results comparing the microbiota of QX-sensitive and QX-resistant families at each location and time point.

	Port Stephens	**Wallis Lake**
January	F = 1.184, p = 0.2233	F = 1.1, p = 0.263
June	F = 1.562, p = 0.0491	F = 1.614, p = 0.0378

* QX-sensitive include F03, F18, F32 and F37 families, QX-resistant includes F022 and F025 families

To further decipher beta diversity patterns between QX-resistant and -sensitive SRO's, STAMP with a Welch's T-Test was used. This analysis identified members of the *Vibrio* (OTU 2, p = 0.003) and *Colwellia* (OTU 3670, p = 0.028) genera with significantly higher relative abundance in the QX-sensitive group from Port Stephens in June (Figure 2-5A). In Wallis Lake, a member assigned as the *Thiohalocapsa* genus (OTU 11899) had a significantly higher relative abundance in QX-sensitive oysters (p = 0.025), whereas OTUs assigned to the *Borrelia* (OTU 651, p = 0.038) and *Endozoicomonas* (OTU 4530, p = 0.047) genera had a significantly higher relative abundance in QX-resistant oysters (Figure 2-5B).

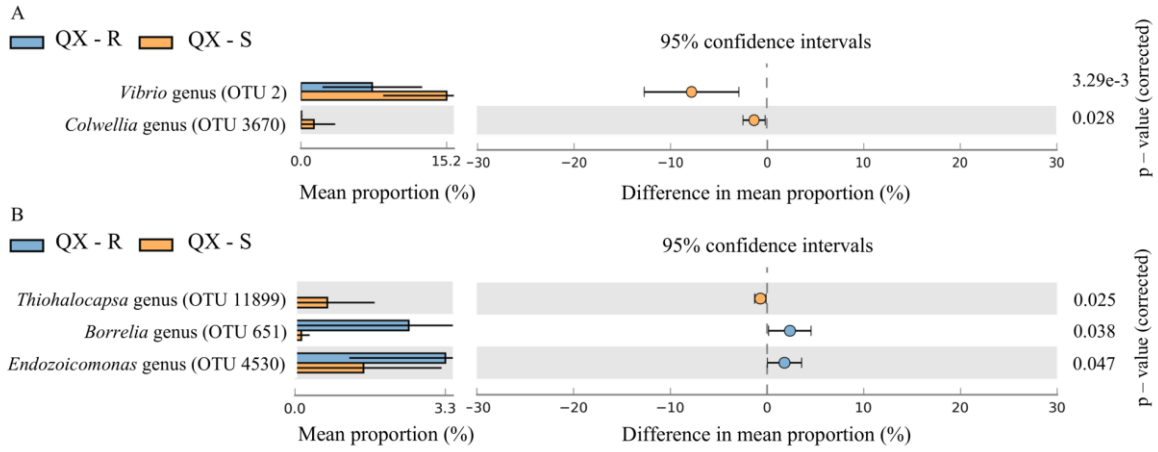


Figure 2-5: Extended error bar plots showing OTUs with a significant difference in relative abundance between the QX-sensitive (QX - S) and resistant groups (QX - R) at Port Stephens (A) and Wallis Lake (B) in June.

2.5 Discussion

This study investigated the influence of location, season and oyster genetics (QX-resistance) on shaping the SRO microbiota. Despite the filter-feeding nature of oysters, our results indicate that the SRO microbiota is highly distinct from the planktonic microbiota within the surrounding seawater. A part of the observed variation may be due to water samples from jetties are slightly different from oyster leases. However, the jetties are facing the lease areas, which are approximately 800 m away. Nevertheless, most likely that the main bacterial patterns in the waters samples would not be varied much across this 800 m. Additionally, it is possible that a part of the observed variation is due to the use of different DNA extraction kits for the oysters and water samples. Nevertheless, this pattern is consistent with previous studies on the microbiota of the Pacific oyster (Lokmer *et al.*, 2016a; Lokmer *et al.*, 2016b).

The microbiota varies between oyster tissues (King *et al.*, 2012; King *et al.*, 2020; Lokmer *et al.*, 2016b) however, some overlap is observed such as the genus *Mycoplasma* which is dominant in the adductor muscle, gill, stomach, digestive gland and haemolymph (Green & Barnes, 2010; King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2020; Wegner *et al.*, 2013). Here, we elected to use the adductor muscle as it allows sampling of the circulatory haemolymph from the sinuses. Overall, the SRO microbiota was dominated by OTUs assigned to the *Candidatus Hepatoplasma*, *Endozoicomonas* and *Mycoplasma* genera. *Candidatus Hepatoplasma* has been found associated with various marine organisms such as starfish (Nakagawa *et al.*, 2017), *Norway lobsters* (Meziti *et al.*, 2012), corals (van de Water *et al.*, 2018) and *starlet sea anemones* (Mortzfeld *et al.*, 2016). However, the function of this bacterium in marine organisms, including SROs, is unknown. *Mycoplasma* is consistently identified in healthy oysters including Eastern oysters, Pacific oyster

and SROs (King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013) suggesting that these bacteria are potentially important for oyster health. Members of the *Endozoicomonas* genus have been found to be associated with numerous marine organisms (Neave *et al.*, 2016) such as sponges (Nishijima *et al.*, 2013; Rua *et al.*, 2014) and corals (Bayer *et al.*, 2013; Ziegler *et al.*, 2016) with members of this genus previously shown to comprise a large proportion of the Indo-Pacific (Roterman *et al.*, 2015; Zurel *et al.*, 2011) and Black-Lipped pearl oyster (Dubé *et al.*, 2019) bacterial communities. In sponges and corals, these bacteria play a role in nitrogen and carbon recycling, provision of proteins to their hosts and production of antibiotics (Neave *et al.*, 2017; Nishijima *et al.*, 2013; Rua *et al.*, 2014) and may suggest a similar role in SROs.

2.5.1 The SRO microbiota is influenced by location

The same oyster families were deployed in Port Stephens and Wallis Lake reducing the influence of genetics as a confounding factor in our analyses and allowing us to investigate whether location or season influence the composition of the SRO microbiota. Consistent with previous studies that have characterised the influence of location on the oyster microbiota (King *et al.*, 2012; Ossai *et al.*, 2017; Roterman *et al.*, 2015; Trabal *et al.*, 2012; Zurel *et al.*, 2011), we observed that SRO microbiota was significantly different between two sites which are approximately 70 km apart and differ in estuarine type (Roy *et al.*, 2001). Data collected in this study identified higher chlorophyll a concentrations and temperature in Port Stephens relative to Wallis Lake. While both estuaries have similar percentages of agricultural land usage in their respective catchments (approximately 30%), Port Stephens has significantly higher sediment and nutrient inputs compared to Wallis Lake (Roper *et al.*, 2011). Given the higher nutrient and sediment loads at Port Stephens, these factors could explain the microbiota variability between the locations. A member of the *Endozoicomonas* genus (OTU 1831) was more abundant in Wallis Lake than in Port Stephens at both sampling times.

In coral species, the anthropogenically influenced coral microbiota (*Pocillopora verrucosa* and *Acropora hemprichii*) was marked by a reduction of *Endozoicomonas* relative abundance (Ziegler *et al.*, 2016), suggesting that the lower relative abundance of this bacteria in SROs at Port Stephens could be related to the higher nutrient and sediment loads.

2.5.2 The SRO microbiota is influenced by season

In a number of marine organisms, including corals (Sharp *et al.*, 2017) and the oyster (Pierce *et al.*, 2016; Zurel *et al.*, 2011) there is evidence for significant temporal heterogeneity in microbiota composition. Consistent with these findings, we observed a significant influence of season (summer versus winter) on the SRO microbiota for both locations. At Port Stephens, seasonal shifts in environmental conditions were dominated by changing temperature, chlorophyll a and conductivity, while at Wallis Lake, seasonal changes in environmental parameters were mostly driven by temperature and pH. Previous studies have characterised the influence of temperature on the oyster microbiota (Lokmer & Wegner, 2015; Pierce *et al.*, 2016) and salinity perturbations have also been observed to influence the oyster microbiota (del Refugio Castañeda Chávez *et al.*, 2005; Larsen *et al.*, 2013). Seasonal shifts in the SRO microbiota were characterised by changes in the relative abundance of several OTUs, including those assigned to the *Candidatus Hepatoplasma* and *Vibrio* genera. Interestingly, we observed inverse patterns for the relative abundance of an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) between the two sampling sites. At Port Stephens, this OTU was significantly more abundant in summer, while at Wallis Lake, it was considerably more abundant in winter. The environmental data collected at the time suggests no similarities between the Port Stephens summer and Wallis Lake winter samples that could explain this pattern (conductivity was similar for these two sampling points but conductivity did not change between the Wallis Lake summer and winter sampling points) and this OTU was

rare or absent in the seawater communities, therefore future studies should increase the suite of environmental parameters collected to explain these patterns. At both locations, a member of the *Vibrio* genus (OTU 2) had a higher relative abundance in winter than in summer. This pattern is interesting given that *Vibrio* typically exhibit preferences for warm water temperatures. However, some *Vibrio* species such as *Vibrio splendidus,* have elsewhere been found to be most abundant during winter and spring (Arias *et al.*, 1999; Pujalte *et al.*, 1999). It is also conceivable that other environmental factors, such as chlorophyll a or nutrient levels, underpinned the higher winter relative abundance of this *Vibrio* species (OTU 2).

2.5.3 The SRO microbiota is influenced by disease resistance

Oyster genetics have been previously shown to influence the Pacific oyster microbiota structure (King *et al.*, 2019c; Wegner *et al.*, 2013), with the microbiota of disease-resistant Pacific oysters showing a significantly different structure to disease-susceptible oysters (King *et al.*, 2019c). However, the influence of genetics on the Pacific oyster microbiota can be superseded by stress, such as temperature perturbations (Wegner *et al.*, 2013). In this study, we observed significant differences of the microbiota between QX-resistant and QX-susceptible oysters, but only in winter (June). This pattern suggests that there is a synergistic interaction of genetics and environmental drivers in shaping the SRO microbiota, which is consistent with previous studies in marine organisms such Pacific oyster (Wegner *et al.*, 2013) and corals (Klaus *et al.*, 2005). While QX disease typically occurs between November to May (Bezemer *et al.*, 2006; Rubio *et al.*, 2013), infections by *M. sydneyi* that cause no mortality (Adlard & Wesche, 2005) have been observed between May to July (Rubio *et al.*, 2013), corresponding to the period where microbiota heterogeneity between resistance groups was observed in this study. This could indicate that the microbiota assemblage prior to the peak mortality period is important and could contribute to QX

disease dynamics, although future studies should consider performing a temporal study to capture possible microbiota dynamics.

A previous study characterising the influence of disease-resistance on Pacific oyster microbiota identified disease-susceptible oysters as having a higher absolute abundance of *Vibrio* species (King *et al.*, 2019c). Interestingly, this pattern is consistent with observations made in this study, where at Port Stephens we observed an over-representation of an OTU assigned to the *Vibrio* genus (OTU 2) in QX-susceptible oysters. *Vibrio* species are commonly implicated as pathogens affecting marine molluscs such as clams, mussels and oysters (Paillard *et al.*, 2004; Travers *et al.*, 2015). For example, *Vibrio* species have a crucial role in summer mortalities of Pacific oysters (de Lorgeril *et al.*, 2018; Garnier *et al.*, 2007; King *et al.*, 2019b; Lemire *et al.*, 2015; Petton *et al.*, 2015; Saulnier *et al.*, 2010; Sugumar *et al.*, 1998) with a non-virulent *Vibrio* community replaced by a pathogenic one (Lemire *et al.*, 2015). Given their role in marine molluscs and other oyster diseases, investigating whether *Vibrio* species influence QX disease dynamics would be of interest. At Wallis Lake, an OTU assigned to the *Endozoicomonas* genus (OTU 4530) was significantly over-represented in the QX-resistant oysters. *Endozoicomonas* bacteria have found to be associated with many marine organisms such as sponges, corals and oysters (Dubé *et al.*, 2019; Neave *et al.*, 2016; Roterman *et al.*, 2015; Zurel *et al.*, 2011). Given the importance of *Endozoicomonas* species in sponges and corals (Neave *et al.*, 2017; Nishijima *et al.*, 2013; Rua *et al.*, 2014), future studies should investigate their potential role in QX-resistant oysters.

2.6 Conclusion

There is emerging evidence that the microbiota of benthic organisms, including oysters, are dynamic and driven by multiple factors, but the impact of location, season and genetics (disease resistance) on the SRO microbiota have not been reported previously. Understanding the factors that drive SRO microbiota composition are pivotal when deciphering the role of the microbiota during disease events, and to explain microbiota shifts prior to, or during, disease. However, this is currently hindered by a paucity of SRO microbiota studies. This study demonstrated that the SRO microbiota assemblage is influenced by location and season, which highlights the importance of performing temporal studies at individual locations as interpreting microbiota patterns from other locations or time points can lead to erroneous microbiota explanations. However, these findings are limited as oysters were collected at only two sampling time points and two locations. Future work should be aimed at sampling across multiple seasons and locations to confirm the influence of these factors on the SRO microbiota. Further, breeding for QX disease resistance (genetics) was found to influence the SRO microbiota although this was only observed in the winter. This sampling time point is before the typical QX disease period, which may indicate that a microbiota shift could be a factor in QX disease dynamics. Overall, these data suggest that there is a synergistic interaction of genetics and environmental drivers in shaping the SRO microbiota.

2.7 Acknowledgements

This research was supported by an Australian Research Council Linkage Project (LP160101785), a Cooperative Research Centre Project (CRC-P 2016-805; Future Oysters), led by the Australian Seafood Industry Pty Ltd in partnership with a number of Australian research organisations and, Ausgem, a research partnership initiated between the University of Technology Sydney and the

New South Wales Department of Primary Industries. VKN was supported by a University of Technology, Sydney – Vietnam International Education Development (UTS - VIED) Scholarship.

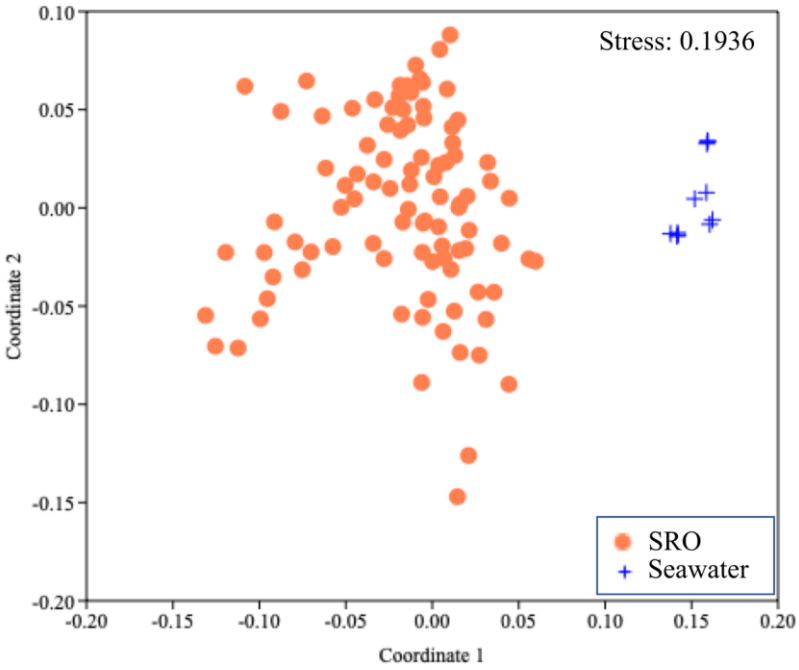
2.8 Supplementary information

Supplementary Table 2-1: Remaining samples for each SRO family and seawater after rarefication to 7,178 reads.

Sample	QX disease resistance level	Port Stephens		Wallis Lake	
		January	June	January	June
F18	Susceptible	3	5	5	4
F22	Resistant	5	5	4	5
F25	Resistant	2	5	4	5
F03	Susceptible	2	5	4	4
F37	Susceptible	3	5	3	5
F32	Susceptible	1	4	3	3
Seawater		2	3	3	3

Supplementary Table 2-2: Kruskal-Wallis ANOVA test of alpha diversity indices between total SRO and total seawater microbiota, including species richness (Chao1), species evenness (Simpson) and species diversity (Shannon).

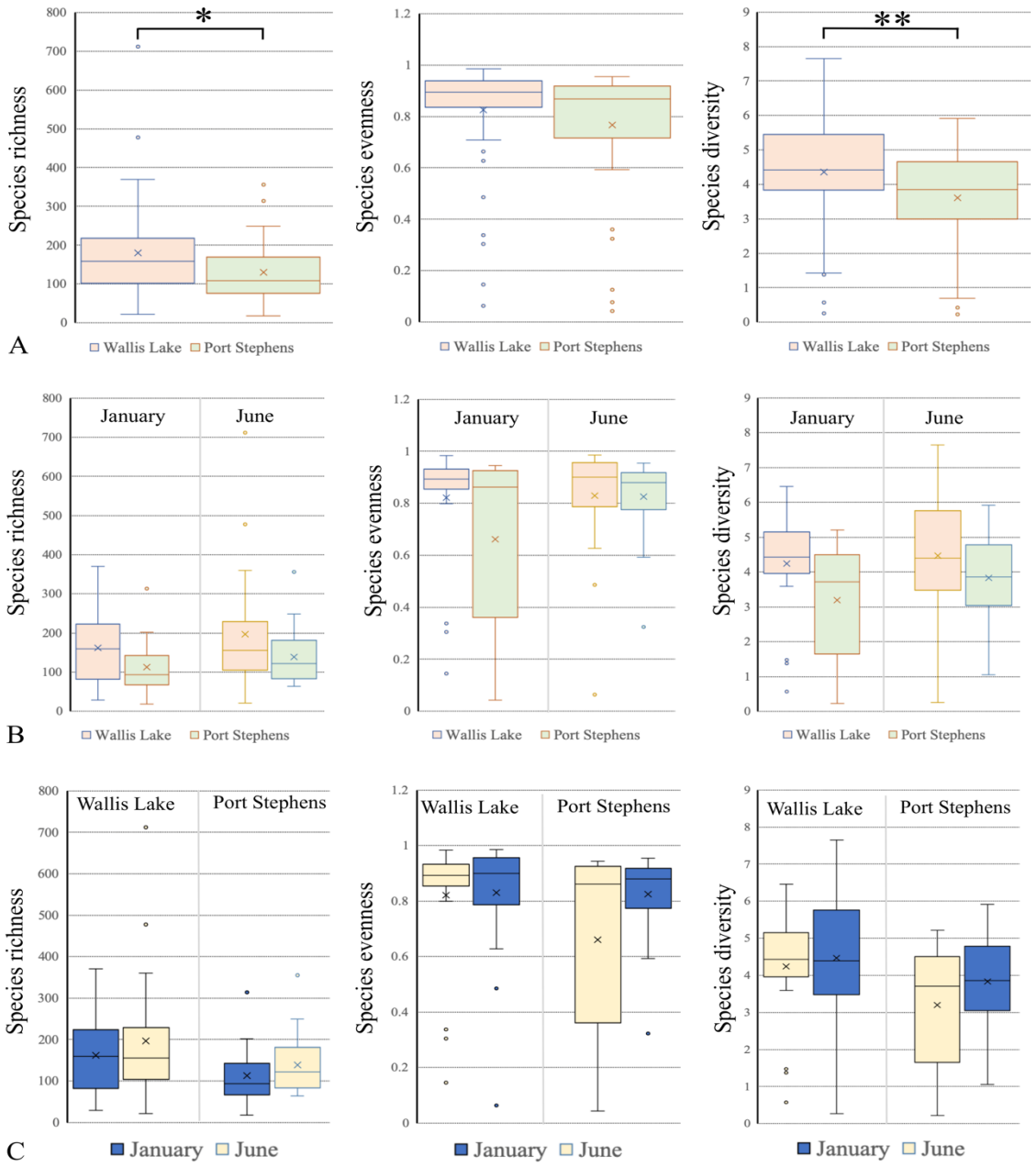
Comparison	H	p-value
Richness of SRO (n =94) vs seawater (n =11)	28.25	1.06E-07
Evenness of SRO (n =94) vs seawater (n =11)	15.64	7.65E-05
Diversity of SRO (n =94) vs seawater (n = 11)	20.06	7.52E-06



Supplementary Figure 2-1: 3D nMDS plot based on Bray-Curtis dissimilarity for comparing the microbiota of 94 oysters and 11 seawater samples and showing separation of the SRO and seawater samples. Axes 1 and 2 are plotted.

Supplementary Table 2-3: SIMPER analysis of the SRO microbiotas between seawater samples. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean SRO	Mean Water
Candidatus Hepatoplasma genus (OTU 14887)	5.736	10.6	0.0481
Candidatus Actinomarina genus (OTU 22961)	4.171	0.0467	7.86
NS5 marine group genus (OTU 5409)	3.641	0.0603	6.75
Endozoicomonas genus (OTU 3829)	2.917	5.38	0.0101
Oceanospirillales order (OTU 12673)	2.554	0.0655	4.82
Endozoicomonas genus (OTU 1831)	2.441	4.5	0
Vibrio genus (OTU 2)	2.255	4.07	0.485
Mycoplasma genus (OTU 14900)	1.885	3.48	0
OM43 clade genus (OTU 6156)	1.867	0.0424	3.47
Arcobacter genus (OTU 6697)	1.787	3.31	0



Supplementary Figure 2-2: Box and whisker plots of species richness, evenness and diversity of total SRO microbiota from Port Stephens and Wallis Lake (A), SRO microbiota from Port Stephens and Wallis Lake at each season (B) and SRO microbiota from January and June and each location (C). The x in the box plot is the mean of the dataset. The single asterisk indicates the statistical significance at $p < 0.05$ and two asterisks show the statistical significance at $p < 0.01$.

Supplementary Table 2-4: Kruskal-Wallis ANOVA test of alpha diversity indices between location and season including species richness (Chao1) species evenness (Simpson) and species diversity (Shannon).

Comparison	H	p-value
Location (January and June)		
Richness in Wallis Lake (n =49) vs Port Stephens (n =45)	4.768	0.02899
Evenness in Wallis Lake (n =49) vs Port Stephens (n =45)	3.769	0.05221
Diversity in Wallis Lake (n =49) vs Port Stephens (n =45)	7.199	0.007294
Location (January)		
Richness in Wallis Lake (n =23) vs Port Stephens (n =16)	2.935	0.08667
Evenness in Wallis Lake (n =23) vs Port Stephens (n =16)	3.134	0.07669
Diversity in Wallis Lake (n =23) vs Port Stephens (n =16)	3.551	0.05951
Location (June)		
Richness in Wallis Lake (n =26) vs Port Stephens (n =29)	2.251	0.1335
Evenness in Wallis Lake (n =26) vs Port Stephens (n =29)	1.201	0.2732
Diversity in Wallis Lake (n =26) vs Port Stephens (n =29)	3.254	0.07126
Season in Wallis Lake		
Richness in January (n =23) vs June (n =26)	0.2508	0.6165
Evenness in January (n =23) vs June (n =26)	0.006421	0.9361
Diversity in January (n =23) vs June (n =26)	0.04013	0.8412
Season in Port Stephens		
Richness in January (n =16) vs June (n =29)	2.6	0.1069
Evenness in January (n =16) vs June (n =29)	0.9918	0.3193
Diversity in January (n =16) vs June (n =29)	0.506	0.4769

Supplementary Table 2-5: SIMPER analysis of the SRO microbiota between Port Stephens and Wallis Lake. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Port Stephens mean	Wallis Lake mean
January and June			
Candidatus Hepatoplasma genus (OTU 14887)	10.05	12	9.27
Endozoicomonas genus (OTU 1831)	4.859	0.0341	8.6
Vibrio genus (OTU 2)	4.309	7.7	0.73
Endozoicomonas genus (OTU 3829)	3.961	3.75	6.88
Mycoplasma genus (OTU 14900)	3.919	6.81	0.423
Arcobacter genus (OTU 6697)	3.611	3.79	2.87
Pseudoalteromonas genus (OTU 8917)	3.323	5.88	0.077
Mycoplasma genus (OTU 12669)	2.896	5.04	0.119
Mycoplasma genus (OTU 14921)	2.865	2.69	3.2
Mycoplasma genus (OTU 14937)	2.69	3.81	1.6
January			
Candidatus Hepatoplasma genus (OTU 14887)	17.66	31.2	0
Arcobacter genus (OTU 6697)	6.468	6.15	6.12
Mycoplasma genus (OTU 14921)	5.095	4.66	6.3
Endozoicomonas genus (OTU 3829)	4.732	5.89	7.02
Endozoicomonas genus (OTU 1831)	3.028	0	5.35
Photobacterium genus (OTU 3)	2.826	0.0871	4.87
Endozoicomonas genus (OTU 6283)	2.761	3.23	4.13
Mycoplasma genus (OTU 14937)	2.737	2.88	3.35
Pseudomonas genus (OTU 12985)	2.304	0.589	3.97
Aquibacter genus (OTU 12017)	2.054	3.63	0.153
June			
Candidatus Hepatoplasma genus (OTU 14887)	9.6	1.43	17.5
Endozoicomonas genus (OTU 1831)	6.38	0.0528	11.5
Vibrio genus (OTU 2)	6.115	11.9	1.31
Mycoplasma genus (OTU 14900)	5.283	9.45	0.218
Pseudoalteromonas genus (OTU 8917)	5.051	9.12	0.111
Mycoplasma genus (OTU 12669)	4.38	7.82	0.0338
Endozoicomonas genus (OTU 3829)	3.424	2.56	6.76
Cobetia genus (OTU 2869)	2.916	5.22	0.00536
Mycoplasma genus (OTU 14937)	2.432	4.32	0.0595
Endozoicomonas genus (OTU 6283)	2.282	2.03	3.78

Supplementary Table 2-6: SIMPER analysis of the SRO microbiota between the two sampling times in Port Stephens and Wallis Lake. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean January	Mean June
Wallis Lake			
Candidatus Hepatoplasma genus (OTU 14887)	10.37	0	17.5
Endozoicomonas genus (OTU 1831)	8.142	5.35	11.5
Endozoicomonas genus (OTU 3829)	4.412	7.02	6.76
Mycoplasma genus (OTU 14921)	3.803	6.3	0.453
Arcobacter genus (OTU 6697)	3.623	6.12	0
Photobacterium genus (OTU 3)	2.96	4.87	0.0177
Endozoicomonas genus (OTU 6283)	2.756	4.13	3.78
Pseudomonas genus (OTU 12985)	2.382	3.97	0.0707
Mycoplasma genus (OTU 14937)	1.995	3.35	0.0595
Endozoicomonas genus (OTU 1993)	1.965	0	3.33
Port Stephens			
Candidatus Hepatoplasma genus (OTU 14887)	16.77	31.2	1.43
Vibrio genus (OTU 2)	6.575	0.00697	11.9
Mycoplasma genus (OTU 14900)	5.612	2.01	9.45
Pseudoalteromonas genus (OTU 8917)	5.008	0	9.12
Arcobacter genus (OTU 6697)	4.553	6.15	2.49
Mycoplasma genus (OTU 12669)	4.288	0	7.82
Endozoicomonas genus (OTU 3829)	3.401	5.89	2.56
Mycoplasma genus (OTU 14937)	3.171	2.88	4.32
Cobetia genus (OTU 2869)	2.854	0.0313	5.22
Mycoplasma genus (OTU 14921)	2.695	4.66	1.6

Supplementary Table 2-7: Kruskal-Wallis ANOVA test of alpha diversity indices between QX-sensitive and QX-resistant groups including species richness (Chao1), species evenness (Simpson) and species diversity (Shannon).

Comparison	H	p-value
Port Stephens in January		
Richness in QX-sensitive (n =9) vs QX-resistant (n =7)	4.26	0.039
Evenness in QX-sensitive (n =9) vs QX-resistant (n =7)	2.692	0.1009
Diversity in QX-sensitive (n =9) vs QX-resistant (n =7)	2.692	0.1009
Wallis Lake in January		
Richness in QX-sensitive (n =15) vs QX-resistant (n =8)	0.6003	0.4385
Evenness in QX-sensitive (n =15) vs QX-resistant (n =8)	0.0375	0.8465
Diversity in QX-sensitive (n =15) vs QX-resistant (n =8)	0.0375	0.8465
Port Stephens in June		
Richness in QX-sensitive (n =19) vs QX-resistant (n =10)	0.6086	0.4353
Evenness in QX-sensitive (n =19) vs QX-resistant (n =10)	1.771	0.1833
Diversity in QX-sensitive (n =19) vs QX-resistant (n =10)	1.895	0.1687
Wallis Lake in June		
Richness in QX-sensitive (n =16) vs QX-resistant (n =10)	2.669	0.1023
Evenness in QX-sensitive (n =16) vs QX-resistant (n =10)	0.1	0.718
Diversity in QX-sensitive (n =16) vs QX-resistant (n =10)	0.5444	0.4606

Supplementary Table 2-8: SIMPER analysis comparing the SRO microbiota of QX-sensitive and QX-resistant groups at Port Stephens and Wallis Lake in June. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean QX-resistant	Mean QX-sensitive
Port Stephens			
Mycoplasma genus (OTU 12669)	9.644	10.9	6.19
Mycoplasma genus (OTU 14900)	9.27	12.7	7.76
Pseudoalteromonas genus (OTU 8917)	6.394	7.59	9.92
Vibrio genus (OTU 2)	6.11	7.24	14.4
Vibrio (OTU 1)	5.662	8.42	0.0667
Mycoplasma genus (OTU 14937)	5.115	5.42	3.74
Cobetia genus (OTU 2869)	4.655	2.24	6.78
Arcobacter genus (OTU 6697)	4.591	6.57	0.345
Marinilabiaceae family (OTU 2173)	3.33	2.42	4.11
Endozoicomonas genus (OTU 6283)	2.644	3.69	1.15
Wallis Lake			
Candidatus Hepatoplasma genus (OTU 14887)	15.86	15.6	18.6
Endozoicomonas genus (OTU 1831)	9.846	11.8	11.3
Endozoicomonas genus (OTU 3829)	4.867	9.2	5.24
Endozoicomonas genus (OTU 1993)	3.495	1.89	4.23
Endozoicomonas genus (OTU 6283)	3.248	5.59	2.64
Gammaproteobacteria class (OTU 6670)	3.003	4.5	0.0679
Endozoicomonas genus (OTU 3483)	2.109	3.33	1.56
Flavobacteriaceae family (OTU 12808)	2.105	0.0111	3.16
Endozoicomonas genus (OTU 1949)	1.975	1.04	2.45
Endozoicomonas genus (OTU 4530)	1.769	3.25	1.4

Chapter 3 Dynamics of the Sydney rock oyster bacterial community prior to and during a QX disease event

Chapter three – Declaration

I declare that the below manuscript meets the below requirements for inclusion as a chapter in this thesis.

- I have contributed more than 50% for the below manuscript.
- The below publication has been formally published and is formatted to adhere to the specific formatting requirements of Aquaculture.

Nguyen, V. K. King, W. L., Siboni, N., Mahbub, K. R., Rahman, Md. H., Jenkins, C., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). Dynamics of the Sydney rock oyster microbiome prior to and during a QX disease event.

Publication status: Under Review.

Date: 10th February 2021

Candidate's signature: Production Note: Signature removed prior to publication.

Dynamics of the Sydney rock oyster bacterial community prior to and during a QX disease event

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3.1 Abstract

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is the most intensively farmed oyster species in Australia however, diseases such as Queensland unknown (QX) disease has caused substantial losses and impeded productivity. The parasite, *Marteilia sydneyi*, is the aetiological agent of QX disease, and like other diseases outbreaks are likely driven by a series of complex environmental and host factors, such as salinity and oyster genetics. A potential but understudied factor in QX disease is the role of the SRO-associated bacterial community, which we sought to examine prior to and during a QX disease outbreak. Using 16S rRNA gene (V1 – V3 region) amplicon sequencing, we examined the bacterial community associated with four SRO families from the breeding program deployed in a region where QX disease occurs, with sampling conducted fortnightly over 22 weeks. A QX outbreak was detected 16 weeks after the first sampling event, with *M. sydneyi* sporonts observed in the digestive gland 2 weeks after pathogen detection. The bacterial community of QX-infected SROs was significantly different from SROs sampled before the outbreak (pre-QX) and from those negative for QX during the outbreak (uninfected) by PCR. The bacterial community shifts associated with QX-infected oysters were principally characterised by a relative abundance increase of OTUs assigned to *Borrelia* and *Candidatus Hepatoplasma* genera and a relative abundance decrease of an OTU belonging to the *Mycoplasma* genus. Since *Mycoplasma* are a common feature of the bacterial community of SROs and other oysters, we propose that there may be an important ecological link between *Mycoplasma* and the health state of SROs. No apparent patterns were observed between the bacterial community of SROs with and without digestive gland sporonts, indicating that QX disease progression does impact the bacterial community but that shifts in bacterial community structure occurred before sporulation in the digestive gland.

Keywords: Bacterial community, *Saccostrea glomerata*, selective breeding, QX disease, *Borrelia*, *Mycoplasma*

3.2 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 northern 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 (NSW) 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 (Kleeman *et al.*, 2002; Wolf, 1979). Once in the digestive gland, the parasite undergoes sporulation, forming mature sporonts containing two tricellular spores (Kleeman *et al.*, 2002; Wolf, 1979) and causing blockage of 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. 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; King *et al.*, 2019a; Raftos *et al.*, 2014). Specifically, low 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 (Butt & Raftos, 2007; Peters & Raftos, 2003) although the full mechanism(s) by which PO is involved in QX resistance is unresolved.

In the more widely studied Pacific oyster (*Crassostrea gigas*) , the bacterial community is emerging as a key factor in disease dynamics (King *et al.*, 2019a; Petton *et al.*, 2015). For example, Pacific oysters with common genetics but varying bacterial communities have different mortality outcomes when challenged with the viral pathogen OsHV-1 (Pathirana *et al.*, 2019). This is likely explained by the fact that OsHV-1 supresses the Pacific oyster immune response 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 bacterial community then they are likely to be less exposed to bacterial infection post OsHV-1 infection (King *et al.*, 2019c; Pathirana *et al.*, 2019; Petton *et al.*, 2015). Additionally, other studies have made links between the oyster bacterial community and disease (King *et al.*, 2019d; Lokmer & Wegner, 2015). For example, one study demonstrated the progressive replacement of a benign *Vibrio* population in the Pacific oyster bacterial community with a virulent population during a mortality outbreak (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 bacterial community associated with SROs might also play a role within QX disease.

Previously, a clone library-based approach demonstrated that the digestive gland bacterial community 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 gene amplicon sequencing, we have recently shown that the bacterial community associated with the SRO adductor muscle is dominated by OTUs assigned to the *Candidatus Hepatoplasma*, *Endozoicomonas* and *Mycoplasma* genera, and that bacterial community structure is significantly influenced by location and season (chapter 2; (Nguyen *et al.*, 2020)). Additionally, we found that the structure of the bacterial community was significantly different between resistant and susceptible QX disease oysters, but only in our winter sampling before the typical QX disease period (late summer or early autumn) with OTUs assigned to the *Mycoplasma*, *Borrelia* and *Endozoicomonas* genera over-represented in the QX resistant SRO bacterial community and OTUs assigned to the *Pseudoalteromonas*, *Vibrio*, and *Candidatus Hepatoplasma* genera over-represented in QX-sensitive SRO bacterial community (chapter 2; (Nguyen *et al.*, 2020)). However, 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 bacterial community in QX disease dynamics is warranted. Here we employed fortnightly sampling to examine temporal shifts in the SRO bacterial community before and during a QX disease event.

3.3 Materials and methods

3.3.1 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 (Dove *et al.*, 2020; Dove *et al.*, 2013a; Nell & Perkins, 2006). SRO families sourced from the NSW Department of Primary Industries SRO Breeding Program were used. Four families (F32, F43, F48 and F67) from 2016 year class were used and only families with intermediate levels of QX disease survival (20 – 50% survival) were selected. Three replicates 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 (Dove *et al.*, 2013a; Nell & Perkins, 2006). 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 by PCR (Peters & Raftos, 2003) and the infection lasts weeks to months (Rubio *et al.*, 2013), fortnightly sampling was considered 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 bacterial communities 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 labelled plastic bags, kept on ice, and immediately transported to the laboratory (within 2 hrs). 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. At each sampling time point, 10 L of seawater was collected at a depth of 10-20 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 2L seawater were filtered with Durapore Membrane Filters (0.22 µm pore size) for DNA extraction. These filtered samples 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.

3.3.2 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). Nutrient analyses (nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) and phosphate (PO_4^{3-})) was conducted at Envirolab Services Pty Ltd (Sydney, NSW, Australia). Chlorophyll a was analysed based on a Spectrophotometric method described previously (Ritchie, 2006).

3.3.3 DNA extractions and 16S rRNA gene amplicon sequencing

Frozen oysters were washed under running tap water to remove debris and allowed to thaw. Using sterile instruments, each oyster was carefully opened using a shucking knife and the oyster flesh

excised and placed into a 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 were amplified by PCR targeting the ribosomal 16S rRNA gene V1–V3 region using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′) primer pair (Lane, 1991; Turner *et al.*, 1999). The PCR cycling conditions were as follows: 94^0C for 2 min, followed by 30 cycles of 94^0C for 30 sec, 50^0C for 30 sec and 72^0C for 30 sec and a final extension at 72^0C for 10 min. Amplicons were sequenced using the Illumina MiSeq platform (2 × 300 bp) at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia). Raw data files in FASTQ format were deposited in the NCBI sequence read archive with the study accession number SRP266167 under Bioproject number PRJNA637460.

3.3.4 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), 1µL PRO2 primer (10 µM stock) and 7 µL water to a total of 20 µL. The PCR cycling conditions were as follows: 94^0C for 2 min, followed by 30 cycles of 94^0C for 30 sec, 50^0C for 30 sec and 72^0C for 30 sec with a final extension of 72^0C 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.

3.3.5 Bioinformatics and statistical analyses

Demultiplexed paired-end reads were combined using FLASH (Magoč & Salzberg, 2011) and filtered by length (471-500 bp) and quality scores (Parameters: maxhomop = 5, maxambig = 0, minlength = 471, maxlength = 500) using Mothur (Schloss *et al.*, 2009). Fragments were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and chimeric and singletons sequences were identified and removed using VSEARCH (Rognes *et al.*, 2016). Taxonomic assignment of OTUs was performed in QIIME version 1.9.1 (Caporaso *et al.*, 2010) using the uclust algorithm (Edgar, 2010) against the SILVA v128 dataset (Quast *et al.*, 2013). Mitochondrial and chloroplast data were filtered out of the dataset. Alpha diversity indices, including Chao1, Simpson and Shannon were calculated using QIIME (Caporaso *et al.*, 2010).

For alpha diversity, differences in species richness (Chao1), species evenness (Simpson) and species diversity (Shannon) between groups were performed using a Kruskal-Wallis test. For beta diversity, samples that had less than 1,000 reads were removed, remaining data normalized using

the proportion method (McKnight *et al.*, 2019) and then OTUs with less than 0.1% relative abundance were filtered out. All analyses were performed with a Bray-Curtis dissimilarity index. Non-metric multidimensional scaling (nMDS) analysis was used to elucidate patterns between sample groups. To determine if bacterial community structures were significantly different, a One-way PERMANOVA with 9999 permutations was used with normalised (square root (x)) data. A Similarity Percentages (SIMPER) test was used to identify the observed dissimilarity of the bacterial communities between groups. These statistical analyses were performed using PAST version 3.24 (Hammer *et al.*, 2001). To determine whether OTUs were significantly different between oyster groups, a Welch's t-test was performed using the STAMP software package version 2.1.3 (Parks *et al.*, 2014). To identify significant associations between environmental variables, QX disease and the SRO bacterial community, a correlation analysis was performed using the MICtools software package with default parameters and the false discovery rate (FDR) method to account for multiple testing (Albanese *et al.*, 2018). Significant correlations with a SpearmanRho ≥ 0.1 and ≤ -0.1 were kept for further analyses (Akoglu, 2018). All OTUs with less than 1% in relative abundance were filtered out prior to analysis and explanatory variables for inclusion in the analysis (i.e. QX infection) were binary transformed. Cytoscape software version 3.6.1 (Su *et al.*, 2014) was used to visualise significant network correlations.

3.4 Results

3.4.1 Sample categorisation and, sequence reads and data filtering

SROs were deployed on the 20th September 2017 with fortnightly sampling commencing on the 8th November 2017. *M. sydneyi* was detected in oysters by PCR approximately 16 weeks after deployment on the 27th of February 2018, signalling a QX outbreak. Before the QX outbreak, a total of 160 oysters had been collected over 16 weeks and were categorised as "pre-QX". During this QX disease outbreak, a total of 140 oysters were collected at four discrete sampling times. Of the 140 oysters, 77 were classified as negative and 63 as positive for *M. sydneyi* presence by PCR (Supplementary Figure 3-1A). Negative and positive samples were categorised as "uninfected" and "QX-infected", respectively. For the *M. sydneyi* PCR-positive SROs, 24 were positive for the presence of mature sporonts in the digestive gland as determined by tissue imprint (Supplementary Figure 3-1B) and categorised as "late QX disease" with those negative categorised as "early QX disease".

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 3-1). 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 bacterial community 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-infected and 62 uninfected (Supplementary Table 3-1). For the 52 QX-infected SROs, 28 were negative (early QX disease) and 24 were positive for mature sporonts in the digestive gland (late QX disease).

3.4.2 The SRO and seawater bacterial community are distinct

Across the dataset, species richness (Chao1), evenness (Simpson) and diversity (Shannon) were significantly higher in the seawater samples compared to the SRO bacterial community (Supplementary Figure 3-2 and Supplementary Table 3-2, $p < 0.001$ for all comparisons). An nMDS plot grouped the SRO and seawater bacterial community separately (Supplementary Figure 3-3), with PERMANOVA confirming that the bacterial communities were significantly different ($F = 53.58$, $p < 0.001$).

SIMPER analysis identified a 98.9% dissimilarity between the seawater and SRO bacterial community. An OTU assigned to the *Mycoplasma* genus (OTU 11355) and *Candidatus Hepatoplasma* genus (OTU 11357) were over-represented in SROs and contributed 9.26% and 6.65% of the dissimilarity, respectively. An OTU assigned to the *Candidatus Actinomarina* genus (OTU 16613) and NS5 marine group genus (OTU 4487) were over-represented in seawater contributing 7.14% and 4.64% of the dissimilarity, respectively (Figure 3-1 and Supplementary Table 3-3).

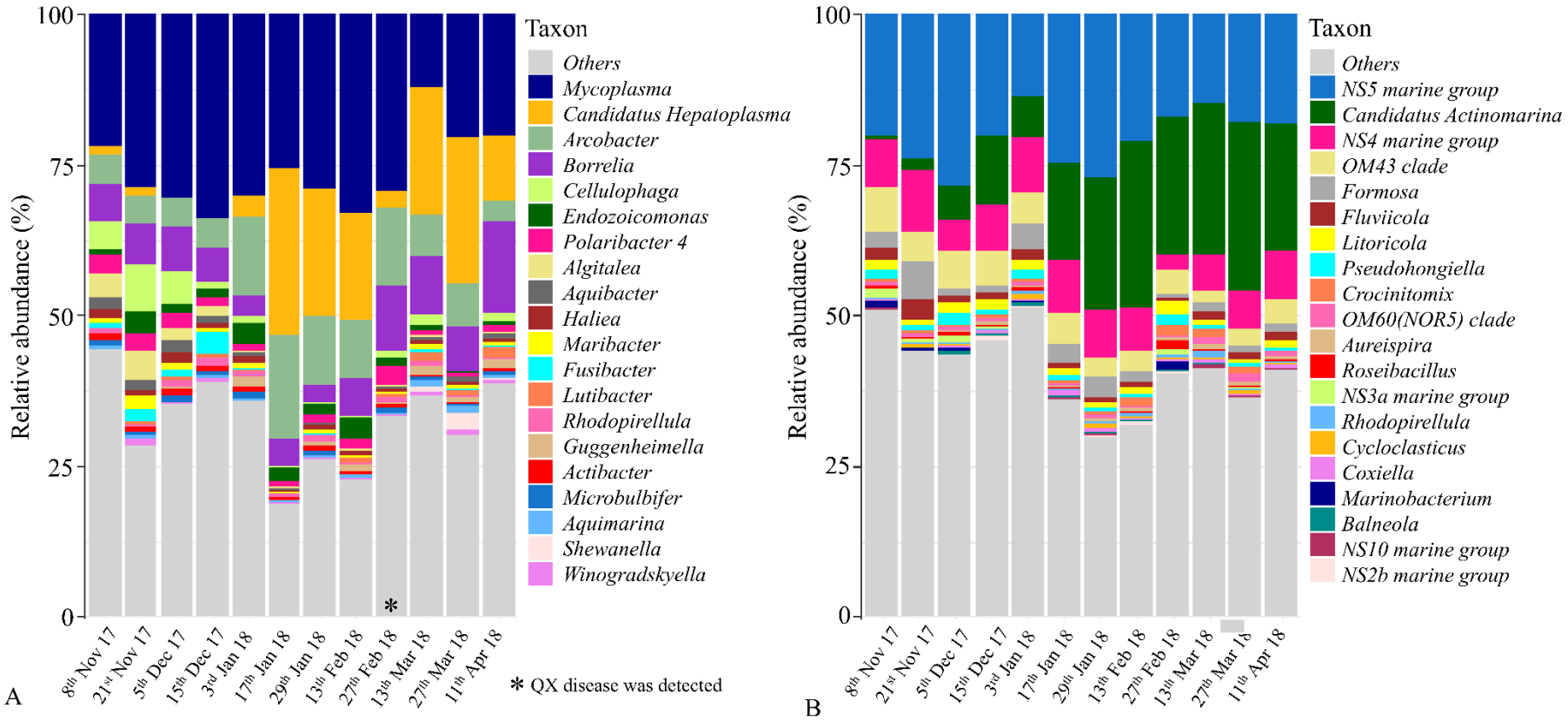


Figure 3-1: Bacterial community of SRO (A) and water samples (B) showing the top 20 dominant and remaining taxa in 12 sampling time points. Data is summarised at the genus level. The asterisk in A indicates the first detection of the QX pathogen.

3.4.3 SRO associated bacterial communities differ according to QX infection state

We examined whether bacterial communities associated with SROs differed according to presence of the *M. sydneyi* parasite by comparing pre-QX, QX-infected and uninfected SRO groups. Alpha diversity indices did not significantly differ between these groups (Figure 3-2 and Supplementary Table 3-4), except for species richness in QX-infected SROs, which was significantly lower than pre-QX SROs (Kruskal-Wallis test, $H = 6.928$, $p = 0.0085$). An nMDS plot showed that the bacterial community structure in QX-infected oysters was more tightly clustered than the pre-QX and uninfected bacterial community (Supplementary Figure 3-4). These patterns were confirmed by statistical analyses with the bacterial community associated with QX-infected SROs significantly different from pre-QX (PERMANOVA $F = 9.423$, $p < 0.001$) and uninfected SROs ($F = 3.282$, $p < 0.001$). Additionally, the bacterial community of pre-QX SROs differed to uninfected SROs ($F = 4.868$, $p < 0.001$). Of the QX-infected SROs, the bacterial communities associated with early and late QX disease groups did not significantly differ ($F = 0.9619$, $p = 0.5099$).

SIMPER analysis revealed that the bacterial community associated with QX-infected SROs was 86.5% and 84.9% dissimilar to pre-QX and uninfected oysters, respectively. Among the QX-infected group, an OTU belonging to the *Candidatus Hepatoplasma* genus (OTU 11357) was substantially over-represented in comparison to pre-QX and uninfected SROs, driving 14.5% and 15.6% of the dissimilarity, respectively (Figure 3-3 and, Supplementary Tables 3-5 and 3-6). An OTU belonging to the *Borrelia* genus (OTU 1) was also over-represented in the QX-infected group responsible for 7.5% and 8.1% of the dissimilarity when compared to the pre-QX and uninfected groups. In contrast, an OTU assigned to the *Mycoplasma* genus (OTU 11355) was over-represented

in the pre-QX and uninfected groups contributing 12.5% and 12.9% of the dissimilarity, respectively (Figure 3-3 and Supplementary Tables 3-5 and 3-6).

A Welch's t-test (STAMP; Parks et al. 2014) identified 175 OTUs with statistically different relative abundances between the pre-QX and QX-infected groups. Of these, 14 OTUs varied by at least 1% relative abundance (Figure 3-4A). In comparisons between the QX-infected and uninfected groups, this approach identified 23 OTUs that differed in relative abundance, with 7 OTUs varying by at least 1% relative abundance (Figure 3-4B). Consistent with the SIMPER results, an OTU assigned to the *Mycoplasma* genus (OTU 11355) was significantly overly represented in the pre-QX and uninfected groups relative to the QX-infected group ($p < 0.001$ and $p = 0.001$, respectively). An OTU assigned to the *Borrelia* genus (OTU 1) was significantly higher in the QX-infected group compared to the pre-QX and uninfected groups ($p< 0.001$ and $p < 0.001$, respectively). Additionally, a member of the *Candidatus Endoecteinascidia* genus (OTU 10028) was significantly higher in the QX-infected group compared to the pre-QX and uninfected groups ($p = 0.046$ and $p = 0.048$, respectively).

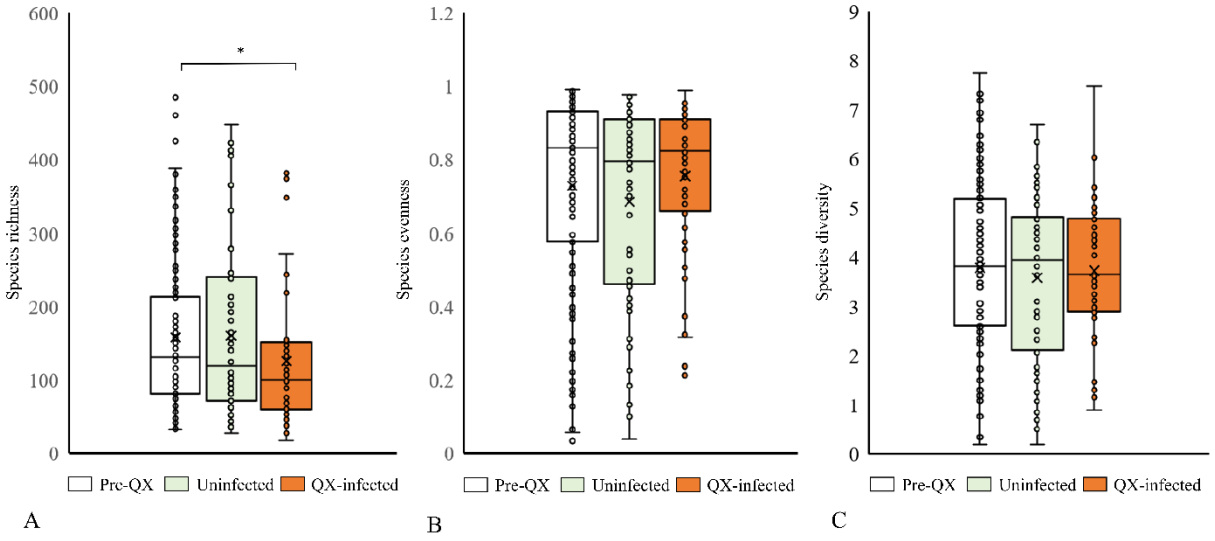


Figure 3-2: Box and whisker plot of species richness (A), evenness (B) and diversity (C) for Pre-QX, uninfected and QX-infected SROs. The x in the box represents the mean of the data set. The asterisk indicates statistical significance at $p < 0.01$.

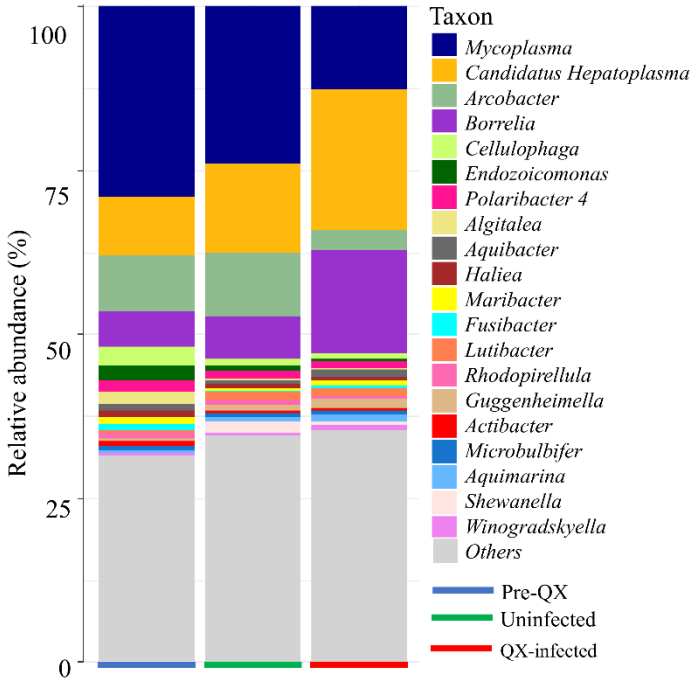


Figure 3-3: Bacterial community composition of SRO groups showing the top 20 dominant and remaining taxa in pre-QX (underlined by the blue bar), uninfected (underlined by the green bar) and QX-infected (underlined by the red bar). Data is summarised at the genus level.

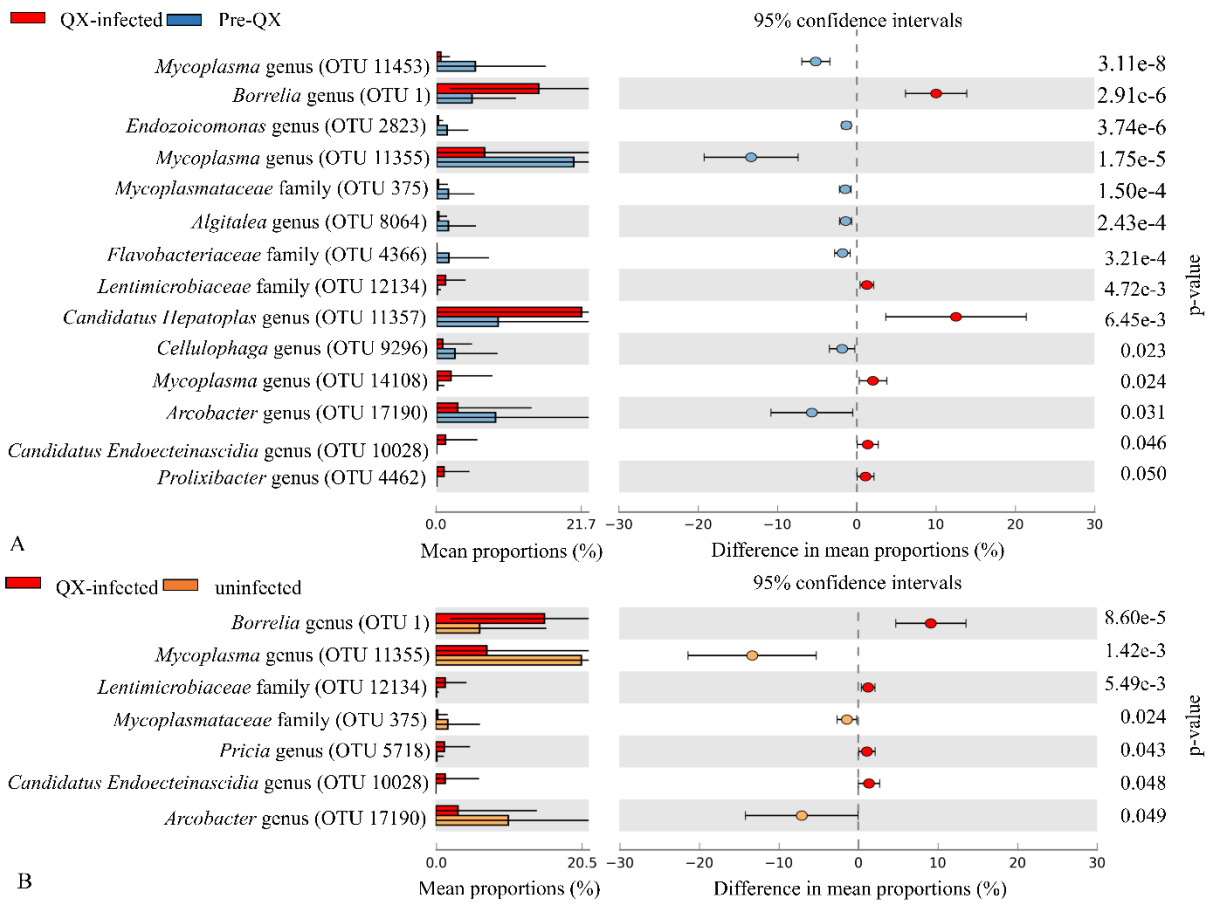


Figure 3-4: Extended error bar plot showing OTUs with a significant difference in relative abundance between the QX-infected and, pre-QX (A) and uninfected (B) groups.

3.4.4 Correlation between environmental variables, QX disease and bacterial community

We investigated correlations between measured environmental variables (Table 3-1) and specific OTUs to QX disease (Figure 3-5A). The MICtool software with Spearman correlation analysis identified 5 environmental parameters were negative and positive correlations with the relative abundance of 12 OTUs (SpearmanRho ≥ 0.1 or ≤ -0.1, $p < 0.05$, Figure 3-5A, Supplementary Table 3-7). Of these environmental parameters, Phosphate displayed the strongest positive correlation with QX infection (SpearmanRho = 0.256 and $p < 0.001$) and pH exhibited the strongest

negative correlation with QX disease (SpearmanRho = -0.24 and $p < 0.001$). Additionally, Phosphate also was negatively correlated with a member of the *Mycoplasma* genus (OTU 11355) (SpearmanRho = -0.13 and $p = 0.004$), and positively correlated with OTU of the *Candidatus Hepatoplasma* genus (OTU 11357) (SpearmanRho = 0.48 and $p < 0.001$) (Figure 3-5B).

QX disease was negatively correlated to the relative abundance of an OTU assigned to the *Mycoplasma* genus (OTU 11355) (SpearmanRho = -0.33 and $p < 0.001$) and were positively correlated with the relative abundance of with OTUs assigned to the *Borrelia* genus (OTU 1) (SpearmanRho = 0.38 and $p < 0.001$) and *Candidatus Hepatoplasma* genus (OTU 11357) (SpearmanRho = 0.31 and $p < 0.001$) to QX disease (Figure 3-5 and Supplementary Table 3-7).

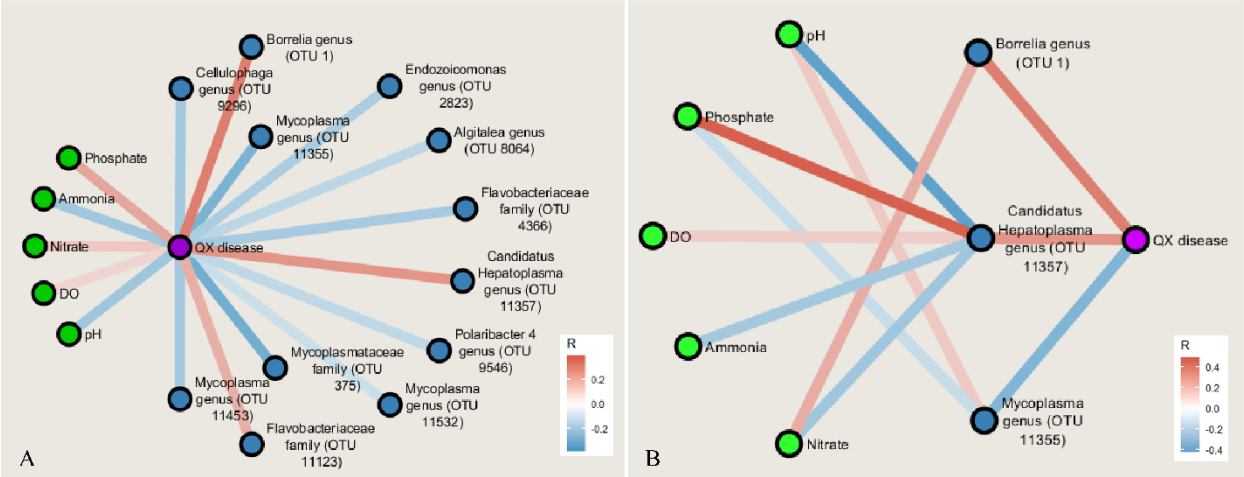


Figure 3-5: Network analysis showing significant correlations of QX disease with specific OTUs with measured environmental variables. Network analysis showing significant correlations of QX disease with specific OTUs with measured environmental variables (A). Correlation of environmental variables and QX disease 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 3-1: 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). Temp: temperature, Conduct: conductivity, Chlo: chlorophyll-a.
^First week QX was detected.

3.5 Discussion

This study characterised SRO associated bacterial communities before and during a QX disease event. Consistent with previous studies in Pacific oysters (Lokmer *et al.*, 2016b) and SROs (Nguyen *et al.*, 2020), our results indicate that SRO associated bacterial communities are highly distinct from bacteria in the surrounding seawater. Over the study period, the SRO bacterial community was dominated by members of the *Mycoplasma, Candidatus Hepatoplasma, Arcobacter* and *Borrelia* genera. These findings are also consistent with our previous SRO bacterial community observations in two locations and over two seasons (Nguyen *et al.*, 2020). Additionally, *Mycoplasma* are dominant in the microbiota of other oysters including Eastern oysters (*Crassostrea virginica*) (King *et al.*, 2012) and Pacific oysters (King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013). Temporal heterogeneity in the oyster bacterial community 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 *Candidatus Hepatoplasma* and *Borrelia* in the uninfected 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 bacterial community of QX-infected SROs was significantly different to QX negative SROs (pre-QX and uninfected), consistent with a previous study that observed the digestive gland bacterial community of QX infected SROs to be different to those of uninfected SROs (Green & Barnes, 2010). Specifically, the QX-infected bacterial community appeared to become more tightly clustered than the pre-QX and uninfected bacterial communities and had reduced species richness. Parasitic infections reduce the filter-feeding capacity of mussels (Stier *et al.*, 2015) which could also be occurring to SROs, thereby reducing the allochthonous input of bacteria and decreasing species richness. In this study,

bacterial community differences according to QX-infection were driven by changes in the relative abundance of OTUs assigned to the *Mycoplasma, Candidatus Hepatoplasma* and *Borrelia*. An OTU assigned to the *Mycoplasma* genus (OTU 11355) was significantly lower in the QX-infected group when compared to pre-QX as well as uninfected QX groups. This result is consistent with a previous study using a clone library approach that characterised the bacterial community associated with the digestive gland in QX infected and non-infected SROs (Green & Barnes, 2010). Additionally, we've previously observed that the relative abundance of *Mycoplasma* is higher in oysters bred for QX resistance compared to QX sensitive oysters in winter, but not summer (Nguyen *et al.*, 2020). As *Mycoplasma* are consistently found to be dominant in healthy oysters such as Eastern oysters (King *et al.*, 2012), Pacific oysters (King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013) and SROs (Green & Barnes, 2010), this genus of bacteria may have an important role in oyster health with its decline an indication of an increased susceptibility to disease. Here, we observed a relative decrease in *Mycoplasma* in uninfected oysters during the QX outbreak compared to the pre-QX oysters with a further relative decrease in SROs that were infected. As there was no difference between the bacterial communities associated with early and late stage QX infected oysters, the decrease in *Mycoplasma* in QX infected oysters does not appear to decrease further as the disease progresses in the SRO. This result is consistent with a previous study showing that the bacterial community of the Eastern oyster did not correlate with infection intensity by the protist pathogen *Perkinus marinus* (Pierce *et al.*, 2016). Our data suggests that a threshold reduction in *Mycoplasma,* 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, it is possible that a relative decrease in *Mycoplasma* 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 *Mycoplasma*, increases in the relative occurrence of OTUs identified as members of the *Candidatus Hepatoplasma* and *Borrelia* were observed in the bacterial community of QX-infected oysters. *Candidatus Hepatoplasma* positively correlated with phosphate and negatively correlated with pH, whereas *Borrelia* positively correlated with nitrate. As was observed with *Mycoplasma*, increases in relative abundance in these OTUs occurred in the uninfected SROs when compared to the pre-QX SROs, with further increases in the QX infected group indicating a possible progressive replacement of *Mycoplasma* with bacteria from these genera. *Candidatus Hepatoplasma* has been reported in various other marine organisms such as starfish, lobster, coral (Meziti *et al.*, 2012; Nakagawa *et al.*, 2017; van de Water *et al.*, 2018) and SROs (Nguyen *et al.*, 2020) however, their function in these marine organisms including SROs, 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. On the other hand, our previous study found an OTU belonging to *Borrelia* was overrepresented in QX resistant oysters, whereas in this study *Borrelia* was associated with QX infected oysters (Nguyen *et al.*, 2020). This should be qualified by the fact that a 16S rRNA alignment show these *Borrelia* OTUs to not be identical and therefore, 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 a member 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 (*Pinctada fucata martensii*) disease (Matsuyama et al. 2017). As with *Mycoplasma,* whether the relative increase in *Candidatus Hepatoplasma* or *Borrelia* facilitates QX infection or their increase is a signature of QX susceptibility requires further investigation.

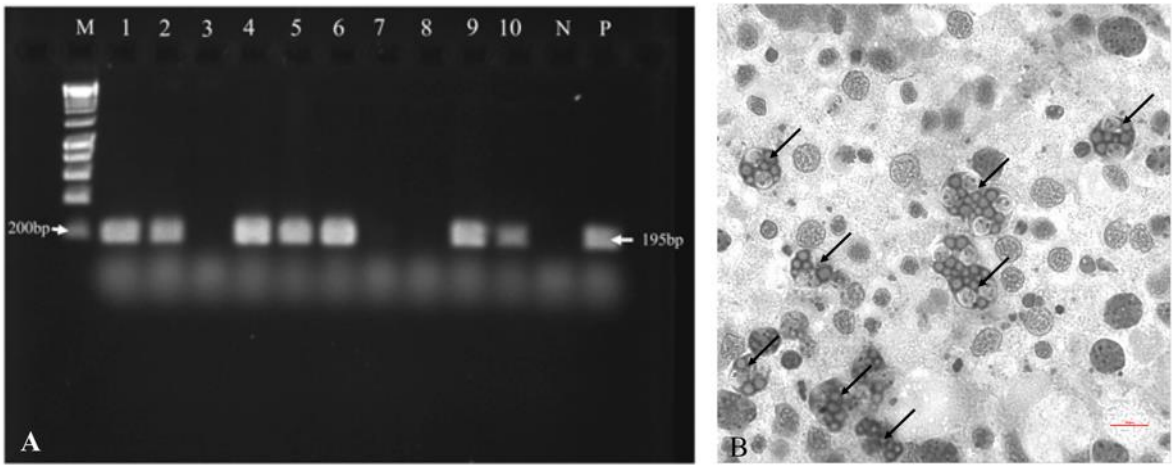
3.6 Conclusion

There is increasing evidence that the oyster bacterial community can play a role in oyster disease, but the impact of the SRO associated bacterial community on QX disease is yet to be resolved. Observing shifts in the bacterial community before and during a disease event is essential when attempting to interpret the interplay between disease and the bacterial community. This study has revealed that the bacterial communities associated with QX-infected oysters are different from those of pre-QX and uninfected oysters. Bacterial community variations were mainly driven by the relative abundance changes of several key bacterial genera including the *Mycoplasma, Candidatus Hepatoplasma* and *Borrelia*, indicating possible roles for these bacteria in QX susceptibility. Additionally, this study has revealed that the bacteria associated with early QX-infected SROs did not differ from those in late QX-infected oysters, implying that the observed shifts in bacterial community composition occur shortly before or at the early stage of disease. This data will aid understanding of the potential involvement of the SRO bacterial community during QX disease and may identify specific bacterial groups that may be useful for monitoring SRO health.

3.7 Acknowledgements

This research was supported by an Australian Research Council Linkage Project (LP160101785), a Cooperative Research Centre Project (CRC-P 2016-805; Future Oysters), led by the Australian Seafood Industry Pty Ltd in partnership with a number of Australian research organisations and, Ausgem, a research partnership initiated between the University of Technology Sydney and the New South Wales Department of Primary Industries. VKN was supported by a University of Technology, Sydney – Vietnam International Education Development (UTS - VIED) Scholarship.

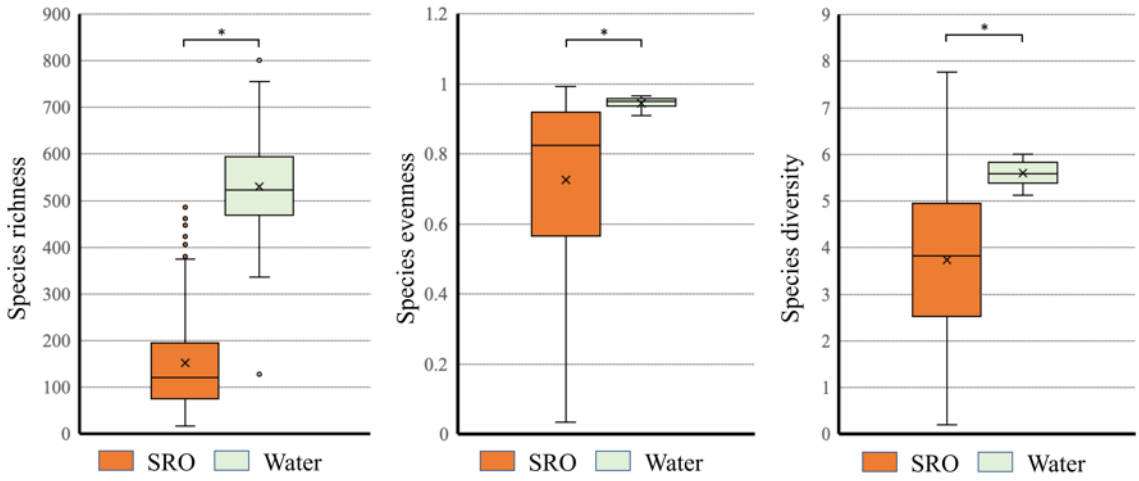
3.8 Supplementary Information



Supplementary Figure 3-1: Gel image of a PCR and tissue imprint for *M. sydneyi* diagnostic. A. Representative gel image of a PCR for *M. sydneyi* showing the results of 10 SROs. N = negative control, P = positive control, M = marker. B. Representative tissue imprint of the SRO digestive gland infected with *M. sydneyi* stained with Rapid diff kit stain showing mature sporonts (arrows). Scale bar = 10 µm.

Supplementary Table 3-1: Sample numbers of SROs after removal of those with less than 1000 reads.

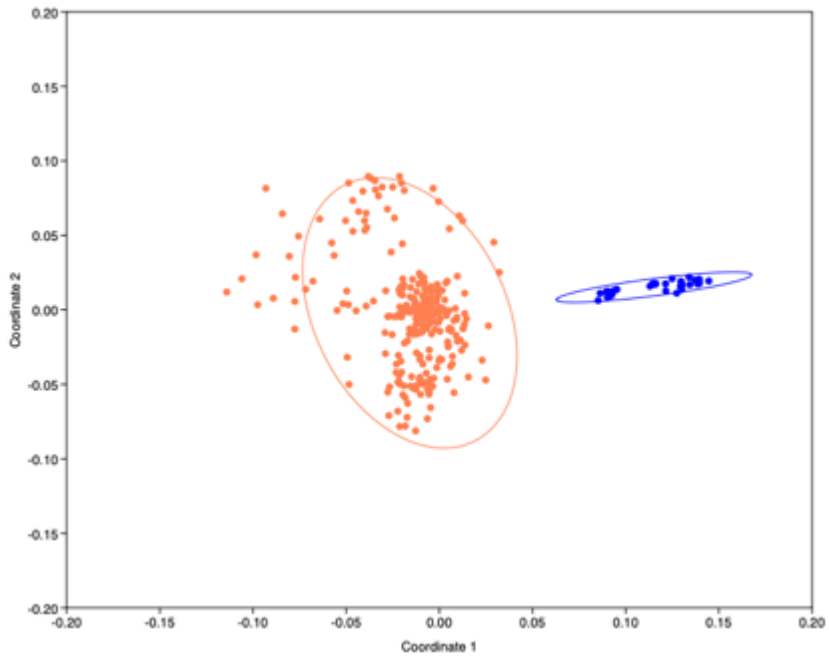
	Total oysters sampled	**Total oysters remaining after filtering**
Pre-QX	160	148
QX-infected	63	52
Uninfected	77	62
Total	300	262



Supplementary Figure 3-2: Box and whisker plot of species richness (A), evenness (B) and diversity (C) for SRO and seawater bacterial communities. The x represents the mean of the data set. The asterisk indicates statistical significance at $p < 0.001$.

Supplementary Table 3-2: Kruskal-Wallis ANOVA test of alpha diversity indices between the SRO and seawater bacterial communities. Species richness (Chao1) species evenness (Simpson) and species diversity (Shannon).

Comparison	H	p-value
Richness of SRO (n = 262) vs seawater (n = 36)	86.97	1.10E-20
Evenness of SRO (n = 262) vs seawater (n =36)	48.01	4.24E-12
Diversity of SRO (n = 262) vs seawater (n = 36)	51.29	7.95E-13



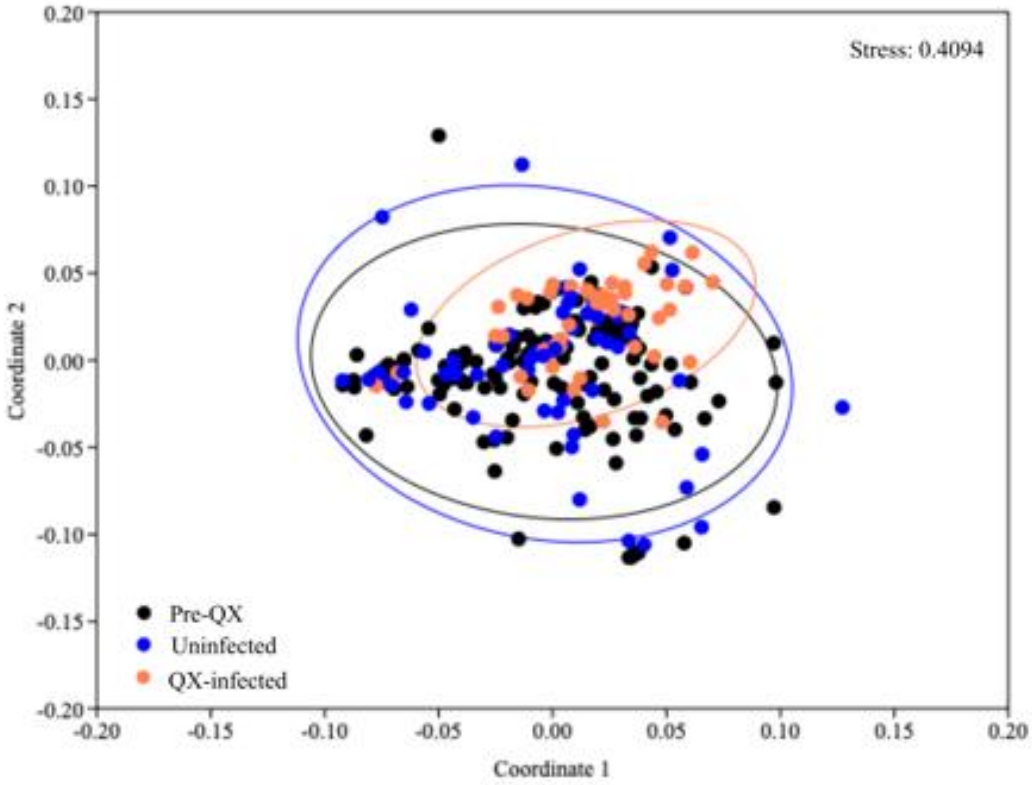
Supplementary Figure 3-3: nMDS plot based on Bray-Curtis dissimilarity comparing the microbiota of 262 oysters and 36 seawater samples and showing separation of the SRO and seawater samples. Axes 1 and 2 are plotted. 95% ellipses are shown. Stress = 0.3511.

Supplementary Table 3-3: SIMPER analysis of the SRO and seawater bacterial communities. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean SRO	Mean Water
Mycoplasma genus (OTU 11355)	9.255	17.4	0.00326
Candidatus Actinomarina genus (OTU 16613)	7.139	0.0463	13.4
Candidatus Hepatoplasma genus (OTU 11357)	6.645	12.5	0
NS5 marine group genus (OTU 4487)	4.641	0.0877	8.79
Arcobacter genus (OTU 17190)	4.151	7.77	0.00281
Borrelia genus (OTU 1)	3.901	7.36	0
SAR86 clade family (OTU 7230)	3.497	0.0761	6.65
OM43 clade genus (OTU 2629)	2.231	0.0302	4.22
Mycoplasma genus (OTU 11453)	1.939	3.64	0
NS4 marine group genus (OTU 9352)	1.735	0.0248	3.28

Supplementary Table 3-4: Alpha diversity comparisons across pre-QX, QX-infected and uninfected bacterial communities including species richness (Chao1) species evenness (Simpson) and species diversity (Shannon).

	H	p-value
Richness		
Pre-QX (n =148) vs Uninfected (n = 62)	0.1781	0.673
Pre-QX (n=148) vs QX-infected (n =52)	6.928	0.0085
Uninfected (n = 62) vs QX-infected (n = 52)	2.913	0.0878
Evenness		
Pre-QX (n =148) vs Uninfected (n = 62)	1.513	0.2188
Pre-QX (n=148) vs QX-infected (n =52)	0.01368	0.9069
Uninfected (n = 62) vs QX-infected (n = 52)	1.037	0.3085
Diversity		
Pre-QX (n =148) vs Uninfected (n = 62)	0.2454	0.6203
Pre-QX (n=148) vs QX-infected (n =52)	0.06855	0.7935
Uninfected (n = 62) vs QX-infected (n = 52)	0.03967	0.8422



Supplementary Figure 3-4: nMDS plot based on Bray-Curtis dissimilarity comparing the microbiota of 148 pre-QX oysters, 62 uninfected oysters and 52 QX-infected oysters and showing tighter clustering of the QX-infected bacterial communities compared to those from the pre-QX and uninfected groups. Axes 1 and 2 plotted. 95% ellipses are shown. Stress = 0.4094.

Supplementary Table 3-5: SIMPER analysis of the SRO bacterial communities between the QX-infected and pre-QX SRO groups. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	**Mean QX-infected**	**Mean Pre-QX**
Candidatus Hepatoplasma genus (OTU 11357)	14.54	21.3	8.97
Mycoplasma genus (OTU 11355)	12.47	6.99	20
Borrelia genus (OTU 1)	7.543	15.1	5.17
Arcobacter genus (OTU 17190)	6.487	3.06	8.58
Mycoplasma genus (OTU 11453)	3.38	0.605	5.64
*Cellulophag*a genus (OTU 9296)	1.948	0.854	2.67
Mycoplasma genus (OTU 14108)	1.333	2.16	0.145
Algitalea genus (OTU 8064)	1.13	0.344	1.72
Mycoplasma genus (OTU 11532)	1.116	0.802	1.31
Mycoplasmataceae family (OTU 375)	1.108	0.282	1.7

Supplementary Table 3-6: SIMPER analysis of the SRO bacterial communities between the QX-infected and uninfected groups. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	**Contrib. %**	**Mean QX-Infected**	**Mean Uninfected**
Candidatus Hepatoplasma genus (OTU 11357)	15.58	21.3	13.6
Mycoplasma genus (OTU 11355)	12.85	6.99	19.7
Borrelia genus (OTU 1)	8.132	15.1	6.09
Arcobacter genus (OTU 17190)	7.253	3.06	9.81
Streptococcus genus (OTU 11123)	2.521	1.62	2.9
Mycoplasma genus (OTU 14108)	1.472	2.16	0.406
Shewanella genus (OTU 1149)	1.237	0.382	1.72
Guggenheimella genus (OTU 14367)	1.151	1.35	0.769
Cellulophaga genus (OTU 9296)	1.144	0.854	1.13
Mycoplasmataceae family (OTU 375)	1.107	0.282	1.63

Supplementary Table 3-7: Mictools identified significant correlations between environmental variables, QX-infected and bacterial communities. The strength of the correlation is the MIC_e estimator of the maximal information coefficient and the Spearman's rank correlation.

Var1	**Var2**	**TICePVal**	**SpearmanRho**	**MICe**
pH	QX disease	1.50E-06	-0.235183	0.321111
pH	*Mycoplasma genus* (OTU 11355)	3.03E-03	0.147208	0.096662
pH	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	-0.404316	0.447098
pH	*Mycoplasma* genus (OTU 11453)	3.78E-04	0.171785	0.146256
pH	*Cellulophaga* genus (OTU 9296)	7.42E-06	0.201472	0.253525
pH	*Polaribacter 4* genus (OTU 9546)	3.19E-04	0.180566	0.141195
pH	*Mycoplasmataceae* family (OTU 375)	1.50E-06	0.340097	0.191098
pH	*Flavobacteriaceae* family (OTU 11123)	1.92E-04	-0.152811	0.151018
pH	*Algitalea* genus (OTU 8064)	9.21E-06	0.182705	0.25009
pH	*Endozoicomonas* genus (OTU 2823)	3.34E-05	0.167359	0.139445
pH	*Flavobacteriaceae* family (OTU 4366)	9.21E-06	0.226591	0.199638
DO	QX disease	1.50E-06	0.110473	0.321111
DO	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	0.135766	0.447098
DO	*Mycoplasma* genus (OTU 11453)	8.81E-05	-0.198085	0.146256
DO	*Cellulophaga* genus (OTU 9296)	1.50E-06	0.195894	0.295548
DO	*Mycoplasma* genus (OTU 11532)	2.74E-03	0.136652	0.087691
DO	*Algitalea* genus (OTU 8064)	1.50E-06	0.258033	0.267426
DO	*Family XIII* family (OTU 11608)	5.09E-04	-0.156773	0.106174
DO	*Flavobacteriaceae* family (OTU 4366)	1.50E-06	0.221091	0.232877
Temperature	*Algitalea* genus (OTU 8064)	1.50E-06	-0.334097	0.267426
Temperature	*Cellulophaga* genus (OTU 9296)	1.50E-06	-0.276631	0.295548

Temperature	*Endozoicomonas* genus (OTU 2823)	1.04E-03	0.116894	0.138374
Temperature	*Family XIII* family (OTU 11608)	1.47E-04	0.156604	0.155144
Temperature	*Flavobacteriaceae* family (OTU 4366)	1.50E-06	-0.284532	0.232877
Temperature	*Mycoplasma* genus (OTU 11453)	4.12E-06	0.228194	0.1552
Temperature	*Mycoplasma* genus (OTU 11532)	4.06E-04	-0.189773	0.091866
Temperature	*Mycoplasmataceae* family (OTU 375)	7.42E-06	0.12943	0.191098
Conductivity	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	0.209775	0.44696
Conductivity	C*ellulophaga* genus (OTU 9296)	2.52E-05	-0.126784	0.252389
Conductivity	*Algitalea* genus (OTU 8064)	4.12E-06	-0.252473	0.248904
Conductivity	*Endozoicomonas* genus (OTU 2823)	1.08E-04	0.124449	0.132741
Conductivity	*Family XIII* family (OTU 11608)	2.21E-02	-0.127195	0.110806
Conductivity	*Flavobacteriaceae* family (OTU 4366)	1.71E-04	-0.108589	0.198777
Nitrate	QX disease	9.21E-06	0.151443	0.27829
Nitrate	*Candidatus Hepatoplasma* genus (OTU 11357)	4.12E-06	-0.231354	0.363364
Nitrate	*Borrelia* genus (OTU 1)	2.53E-05	0.233855	0.149747
Nitrate	*Cellulophaga* genus (OTU 9296)	1.50E-06	0.260755	0.187637
Nitrate	*Polaribacter 4* genus (OTU 9546)	1.43E-03	0.108949	0.109901
Nitrate	*Mycoplasmataceae* family (OTU 375)	1.52E-04	-0.240421	0.128286
Nitrate	*Algitalea* genus (OTU 8064)	5.20E-06	0.259354	0.195047
Nitrate	*Endozoicomona*s genus (OTU 2823)	3.34E-04	-0.140059	0.15302
Nitrate	*Flavobacteriaceae* family (OTU 4366)	4.28E-05	0.214131	0.139136
Ammonia	QX disease	1.50E-06	-0.230525	0.318801
Ammonia	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	-0.220919	0.35888
Ammonia	*Mycoplasma* genus (OTU 11453)	2.53E-05	0.134928	0.138304
Ammonia	*Cellulophaga* genus (OTU 9296)	1.50E-06	0.294369	0.249948
Ammonia	*Polaribacter 4* genus (OTU 9546)	6.00E-04	0.156712	0.103566
Ammonia	*Flavobacteriaceae* family (OTU 11123)	6.68E-04	-0.117107	0.138616

Ammonia	*Algitalea* genus (OTU 8064)	1.50E-06	0.221032	0.245722
Ammonia	*Flavobacteriaceae* family (OTU 4366)	1.50E-06	0.218199	0.195927
Phosphate	QX disease	1.50E-06	0.256159	0.321111
Phosphate	*Mycoplasma* genus (OTU 11355)	4.00E-03	-0.126947	0.11536
Phosphate	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	0.47678	0.447098
Phosphate	*Cellulophaga* genus (OTU 9296)	1.50E-06	-0.4111	0.295548
Phosphate	*Polaribacter 4* genus (OTU 9546)	2.27E-04	-0.155591	0.12566
Phosphate	*Mycoplasmataceae* family (OTU 375)	3.09E-04	-0.162255	0.191098
Phosphate	*Mycoplasma* genus (OTU 11532)	1.47E-03	-0.216891	0.104514
Phosphate	*Flavobacteriaceae* family (OTU 11123)	1.50E-06	0.233974	0.151018
Phosphate	*Algitalea* genus (OTU 8064)	1.50E-06	-0.463778	0.267426
Phosphate	*Endozoicomonas* genus (OTU 2823)	2.00E-03	-0.140369	0.144835
Phosphate	*Flavobacteriaceae* family (OTU 4366)	1.50E-06	-0.410508	0.232877
Chlorophyll-a	*Mycoplasma* genus (OTU 11453)	6.04E-05	-0.241065	0.146256
Chlorophyll-a	*Cellulophaga* genus (OTU 9296)	8.24E-04	0.123293	0.253525
Chlorophyll-a	*Mycoplasmataceae* family (OTU 375)	3.05E-04	-0.114259	0.191098
Chlorophyll-a	*Mycoplasma* genus (OTU 11532)	5.34E-04	0.178426	0.119964
Chlorophyll-a	*Algitalea* genus (OTU 8064)	6.94E-04	0.158673	0.25009
Chlorophyll-a	*Flavobacteriaceae* family (OTU 4366)	6.67E-04	0.132813	0.199638
QX disease	*Mycoplasma* genus (OTU 11355)	1.50E-06	-0.32586	0.176362
QX disease	*Candidatus Hepatoplasma* genus (OTU 11357)	1.50E-06	0.310233	0.155186
QX disease	*Borrelia* genus (OTU 1)	1.50E-06	0.381101	0.204946
QX disease	*Mycoplasma* genus (OTU 11453)	9.21E-06	-0.22356	0.135512
QX disease	*Cellulophaga* genus (OTU 9296)	1.92E-04	-0.221664	0.097147
QX disease	*Polaribacter 4* genus (OTU 9546)	1.35E-02	-0.149997	0.097341
QX disease	*Mycoplasmataceae* family (OTU 375)	1.50E-06	-0.349447	0.198049
QX disease	*Mycoplasma* genus (OTU 11532)	4.38E-02	-0.116075	0.07005

QX disease	*Flavobacteriaceae* family (OTU 11123)	4.79E-04	0.22631	0.141314
QX disease	*Algitalea* genus (OTU 8064)	6.27E-03	-0.156114	0.083341
QX disease	*Endozoicomona*s genus (OTU 2823)	8.84E-04	-0.201124	0.08802
QX disease	*Flavobacteriaceae* family (OTU 4366)	6.55E-05	-0.212611	0.073277

Chapter 4 Changes in the abundance of selected core microbiota in the Sydney rock oyster before and during a QX disease outbreak

4.1 Abstract

We sought to examine the variability in the microbiota of Sydney rock oysters (SROs; *Saccostrea glomerata*) across families and identify taxa indicative of healthy or QX-infected SROs. Using 16S rRNA gene (V1 – V3 region) amplicon sequencing, we examined the SRO microbiota associated with four SRO families deployed in Georges River, New South Wales (NSW) Australia where Queensland unknown (QX) disease is seasonally recurrent. Sampling was conducted fortnightly over 22 weeks with a QX disease event detected 16 weeks after the first sampling time point. Overall, the microbiota of SRO was significantly different between families with the variation mainly driven by OTUs assigned to the *Mycoplasma*, *Arcobacter* and *Candidatus Hepatoplasma* genera. OTUs assigned to *Mycoplasma, Candidatus Hepatoplasma* and *Borrelia* genera were identified as core and linked to the QX disease event. Of these, shifts in the *Mycoplasma* and *Borrelia* OTUs were consistent across all families and were selected for qPCR to measure absolute abundance. The absolute abundance of *Mycoplasma* increased in the QX-infected group compared to the pre-QX group, contrary to the 16S rRNA gene sequencing results possibly indicating that one (or more) *Mycoplasma* OTU(s) is replaced by other *Mycoplasma* OTUs. Additionally, an increase in the absolute abundance of *Borrelia* in QX-infected SRO compared to pre-QX SRO was observed which is consistent with the 16S rRNA gene sequencing data and therefore, may be a good taxa for assessing the health of SROs or their susceptibility to QX disease.

Keywords: Core microbiota, Sydney rock oyster, QX disease, *Mycoplasma*, *Borrelia*.

4.2 Introduction

In many higher marine organisms, the microbiota can benefit their host in multiple ways such as assisting nutrient uptake (Ainsworth *et al.*, 2015) or producing antimicrobial substances that inhibit pathogens (Bakkiyaraj *et al.*, 2013; Desriac *et al.*, 2014; Shnit-Orland & Kushmaro, 2009). However, the microbiota can also harbour opportunistic or pathogenic bacteria that can cause disease under the right conditions (Roder *et al.*, 2014). Despite the variability in microbiota composition, there are often core members that are conserved over geographic scales (Ainsworth *et al.*, 2015) and time (Aronson *et al.*, 2017). A core microbiota is defined as members that are shared among microbiota from similar habitats (Shade & Handelsman, 2012). Based on this definition, a core microbiota can be identified based on shared OTU occurrence over time and across communities (Hernandez-Agreda *et al.*, 2018; Huse *et al.*, 2012; King *et al.*, 2012; King *et al.*, 2019c; Lawson *et al.*, 2018; Schmitt *et al.*, 2012), and OTUs present from 50 % to 100 % of samples can be considered as core microbiota (Hernandez-Agreda *et al.*, 2016; ; Lawson *et al.*, 2018; Schmitt *et al.*, 2012). Depending on the research question, an appropriate definition of core microbiota is applied to identify the stable and consistent members across complex microbial assemblages (Shade & Handelsman, 2012). Due to their conservation across habitats, core microbiota are thought to have an important functional role and act as good targets for studies investigating host-microbial interactions (Astudillo-Garcia *et al.*, 2017).

In Pacific oysters (*Crassostrea gigas*), there is increasing evidence for the role of the microbiota in health and disease (King *et al.*, 2019b; King *et al.*, 2019c; Lokmer & Wegner, 2015; Petton *et al.*, 2015; Wegner *et al.*, 2013) with shifts in microbiota composition becoming linked to disease (de Lorgeril *et al.*, 2018; King *et al.*, 2019b). However, the microbiota of

oysters are influenced by multiple factors such as geographic location and associated environmental parameters, time and genetics (King *et al.*, 2019c; Lokmer *et al.*, 2016b; Nguyen *et al.*, 2020; Trabal *et al.*, 2012; Wegner *et al.*, 2013). Because of the diversity, complexity and high dynamic nature of microbiota in the oysters, studies in the Eastern oyster (King *et al.*, 2012) and Pacific oyster (King *et al.*, 2019c; Lasa *et al.*, 2019) have characterised the core microbiota to assist in understanding the functional role of specific taxa. One previous study identified OTUs from the the *Winogradskyella* genus and the *Bradyrhizobiaceae* family as core in Pacific oyster families resistant to OsHV-1 (King *et al.*, 2019c) and suggested that these might may play a role in OsHV-1 disease resistance (King *et al.*, 2019c).

The factors shaping the microbiota of the Sydney rock oyster (SRO; *Saccostrea glomerata*) are only beginning to be studied including in the context of the devastating Queensland unknown (QX) disease (Chapter 2; (Nguyen *et al.*, 2020). In chapter 2, we observed that the SRO microbiota is dominated by OTUs assigned to the *Endozoicomonas*, *Mycoplasma* and *Candidatus Hepatoplasma* genera (Chapter 2; (Nguyen *et al.*, 2020) and that the microbiota of SROs is influenced by location, season and genetics associated with selectively breeding for QX disease resistance (Chapter 2; (Nguyen *et al.*, 2020)). QX disease resistance was a factor in winter and not in the summer however, winter is before the typical QX disease infection period. OTUs belong to the *Mycoplasma*, *Borrelia* and *Endozoicomonas* genera were over-represented in the microbiota of QX resistant SROs whereas, OTUs assigned to the *Pseudoalteromonas, Vibrio*, and *Candidatus Hepatoplasma* genera were over-represented in QX-sensitive SROs (Chapter 2; (Nguyen *et al.*, 2020)). In chapter 3, we observed that the microbiota of SROs infected with the QX pathogen *Marteilia sydneyi* was significantly different from SROs sampled prior to the outbreak (pre-QX) and from those negative for QX during the outbreak (uninfected). OTUs belonging to the *Borrelia* and *Candidatus*

Hepatoplasma genera were over-represented in QX infected oysters, while OTUs assigned to the *Mycoplasma* genus were over-represented in pre-QX and uninfected oysters. Here, it is hypothesised that there are key indicator taxa that can be used to differentiate QX-diseased and healthy Sydney rock oysters. To test this hypothesis, we examined the variability in the SRO microbiota across four SRO families and then identify core OTUs for further analyses. Finally, we develop and trial two qPCR methods for measuring the absolute abundance of two core OTUs before and during a QX disease event.

4.3 Methods

4.3.1 Experimental design and sampling

The experimental design and sampling are provided in detail in chapter 3. Briefly, four families within intermediate QX resistance (Table 1) were deployed on the 20th of September 2017 in Georges River, NSW Australia (33°59'19"S 151°03'21"E) 7 weeks before the commencement of sampling. Oysters were collected every fortnight over 22 weeks with a QX outbreak detected 16 weeks into the experiment. Oysters were randomly collected from cultivation trays, placed into a labelled plastic bag and kept on ice for transport within one hour to the laboratory and processed as described in chapter 3.

Table 4-1: 2016-year class Sydney rock oyster average family survival (n = 3, ± SD) following exposure to QX disease at Lime Kiln Bar, Georges river.

Oysters were deployed to Lime Kiln Bar on 8 February 2017 and oyster survival was counted on 9 July 2018.

Family line	Average survival (%)
F32	20 ± 10.6
F43	33 ± 3
F48	41 ± 3.6
F67	41.3 ± 11

4.3.2 DNA extractions, 16S rRNA gene amplicon sequencing and bioinformatics

DNA extractions, 16S rRNA gene amplicon sequencing and bioinformatics methodologies are provided in detail in chapter 3. Briefly, approximately 25-50 mg of adductor muscle tissue was used for DNA extraction using the Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. Extracted DNA was amplified by PCR targeting the 16S rRNA gene V1–V3 region using the 27F (5′-AGAG

TTTGATCMTGGCTCAG-3′) and 519R (5′- GWATTACCGCGGCKGCTG-3′) primer pair (Lane, 1991; Turner *et al.*, 1999). Amplicons were sequenced using the Illumina MiSeq platform (2 × 300 bp) at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia).

Demultiplexed paired-end reads were combined using FLASH (Magoč & Salzberg, 2011) and trimmed using Mothur (Schloss *et al.*, 2009). Fragments were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and chimeric and singletons sequences were identified and removed using VSEARCH (Rognes *et al.*, 2016). Taxonomic assignment of OTUs was performed in QIIME version 1.9.1 (Caporaso *et al.*, 2010) using the UCLUST algorithm (Edgar, 2010) against the SILVA v128 dataset (Quast *et al.*, 2013). Mitochondrial and chloroplast data were filtered out of the dataset. Alpha diversity indices, including Chao1, Simpson and Shannon were calculated using QIIME (Caporaso *et al.*, 2010).

4.3.3 PCR detection of the QX pathogen *Marteilia sydneyi*

PCR detection of *M. sydneyi* are given detail in chapter 3 using the primers LEG1 (5'-CGATCTGTGTAGTCGGATTCCGA) and PRO2 (5'-TCAAGGGACATCCAACGGTC) as previously developed by Kleeman and Adlard (2000).

4.3.4 Core microbiota analysis

We used the panbiom.py analysis described in Kahlke (2017) to identify the core microbiota for each family within each of the pre-QX, uninfected and QX-infected groups. No core microbiota was identified when the replicate threshold (-r parameter) was set at 100 % or 90

% therefore, the replicate threshold was set to 80 % defined as OTUs present in at least 80% of samples. When showing the core microbiota shared between families within each group, the online tool of Bioinformatics & Evolutionary Genomics was used to generate Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/).

4.3.5 Quantitative PCR (qPCR)

In order to quantify *Borrelia* genus OTU 1 and *Mycoplasma* genus OTU 11355, the sequence of these OTUs were extracted from the 16S rRNA gene sequencing data (Supplementary Table 4-1). These sequences were aligned against previously published *Borrelia* and *Mycoplasma* primers (Supplementary Table 4-2) using Molecular Evolutionary Genetics Analysis (MEGA) software version 10.1.1 (Kumar *et al.*, 2018) to test the compatibility of these primers to these sequences. For the *Borrelia* primers, the alignment results showed the forward S0775 primer to have only one single nucleotide mismatch to the sequence of *Borrelia* genus OTU 1 (Supplementary Figure 4-1A). We slightly modified the primer to produce BoR_F 5'-TCCTACGGGAGGCAGCAGC-3' (modified nucleotide shaded grey). As the reverse primer (S0776) target region was outside of the *Borreli*a genus OTU 1 sequence (Supplementary Figure 4-1A), we designed a new reverse primer from our *Borrelia* genus OTU 1 sequence using the Primer-BLAST tool (Ye *et al.*, 2012) setting BoR_F as the forward primer. From this, 10 possible primer pairs were generated and when these were BLASTED to existing sequences in the NCBI database, one pair (BoR_F: 5'-TCCTACGGGAGGCAGCAGC-3' and BoR_R: 5'- TCCTAGCTTACCGTCATCGCA-3) consistently aligned with sequences from *Borrelia* species and were selected for qPCR assay development. The primer pair generates a product of 156 bp in length.

For the published *Mycoplasma* primers, none annealed to our *Mycoplasma* genus OTU 11355 sequence due to their targeting of regions outside V1-V3. We made the assumption that if our *Mycoplasma* genus (OTU 11355) sequence was 99-100% match to a sequence in the NCBI GenBank database, we could use this to assess the published primers and modify them where necessary. On BLASTing our *Mycoplasma* genus (OTU 11355) sequence against NCBI, we retrieved a sequence with 100% coverage and 99.79% identity to a sequence with accession number FM995178.1. We then used this sequence for aligning to the previously published *Mycoplasma* primers (Supplementary Table 4-2) with the alignment showing primers F4 and R4 having one and four single nucleotide mismatches respectively (Supplementary Figure 4-1B). We modified the F4 and R4 primers to produce Myco_F: 5'-CTCCGCCTGAGTAGTATGC-3' and Myco_R: '5-CACCTGTCTCAATGTTAACCTC-3' (modified nucleotides shaded grey) generating a product of 169 bp. We BLASTED the primer pair and they showed consistent alignment with sequences catalogued as *Mycoplasma* species and were therefore selected for qPCR assay development.

Quantitative PCR (qPCR) was conducted using an epMotion 5075l Automated Liquid Handling System integrated with a Bio-Rad CFX384 Touch Real-Time PCR Detection System. All analyses were performed with three technical replicates with a standard curve using synthetic oligonucleotides as negative controls. Each reaction mixture contained: 2.5 μL iTaq Universal SYBR Green Supermix, 0.2 μL of each 10 μM forward and 10 μM reverse primers, 1 μL of template DNA, and 1.1 μL of sterile water. For the *Borrelia* qPCR, cycling conditions were 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 53°C for 30 seconds, 72°C for 30 seconds and data collection at 77^0C for 15 seconds. For the *Mycoplasma* qPCR, cycling conditions were 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 47.5°C for 30 seconds and data collection at 72°C for 30 seconds. A melting

curve was added to the end of every run to confirm the presence of a single PCR product. All qPCR data was 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.

4.3.6 Statistical analyses

Comparison of alpha diversity, including richness (Chao1), species evenness (Simpson) and species diversity (Shannon) between families were performed using a Kruskal-Wallis test. Non-metric multidimensional scaling (nMDS) was used to visualise distance matrices between sample groups. To determine significantly different microbiota structures, one-way PERMANOVA with 9999 permutations was used. The relative abundance was normalised (square root (x) before running a One-way PERMANOVA. Similarity Percentages (SIMPER) was used to identify the observed dissimilarity of the microbiota between groups. Beta diversity analyses were performed with a Bray-Curtis dissimilarity index. Comparisons of 16S rRNA gene copies (16S rRNA qPCR) were performed with a Kruskal-Wallis statistical test. All comparisons, including Kruskal-Wallis, nMDS, PERMANOVA and SIMPER were performed in the PAST statistical environment (Hammer *et al.*, 2001).

4.4 Results

4.4.1 Sequence reads and data filtering

Following amplicon sequencing of the 300 oyster samples and removal of samples with less than 1,000 reads, a total of 1,949,501 reads were obtained from 262 SROs. After data filtering, a total of 3,492 OTUs were identified across the entire dataset. A total of 148 oysters were pre-QX with the remaining 114 oysters collected after detection of QX disease consisting of 52 QX-infected and 62 uninfected. The numbers of remaining oysters for each family (F32, F43, F48 and F67) ranged from 63 to 69 (Table 4-2).

Table 4-2: Number of oysters sampled and remaining after removal of those with less than 1000 reads for each family within the pre-QX, uninfected and QX-infected groups.

Family	Pre-QX		Uninfected		QX-infected		Total	
	S	R	S	R	S	R	S	R
F32	40	36	9	6	26	21	**75**	**63**
F43	40	38	25	20	10	9	**75**	**67**
F48	40	36	21	17	14	10	**75**	**63**
F67	40	38	22	19	13	12	**75**	**69**

S: oysters sampled, R: oysters remaining after filtering

4.4.2 Variability of the SRO microbiota across families

We examined whether the SRO microbiota for the pre-QX, uninfected and QX-infected groups differed across the four families. Overall, alpha indices did not significantly differ between the families in each group (Kruskal-Wallis test, $p>0.05$; Figure 4-1 and Supplementary Table 4-3), 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) (Figure 4-1 and Supplementary Table 4-3). No clear dissimilarity of the microbiota composition between families was observed in a 3D nDMS plots (Figure 4-2) however, statistical analyses showed that most of the SRO microbiota of the families differed from one another in each group (Table 4-3).

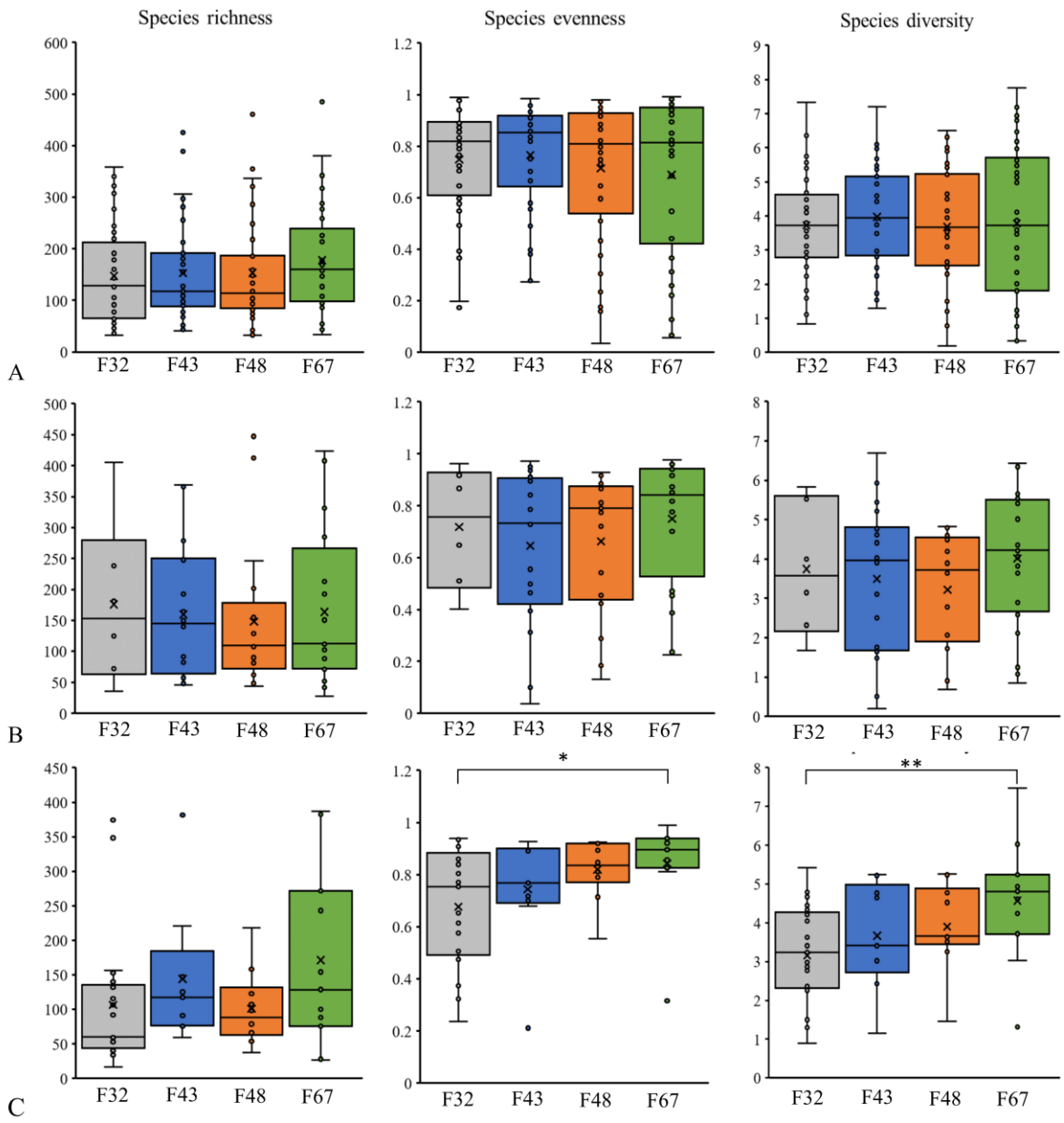


Figure 4-1: Box and whisker plots of species richness (Chao 1), evenness (Simpson) and diversity (Shannon) of each SRO family within the pre-QX (A), uninfected (B) and QX-infected groups (C). The x represents the mean of the data set. The single and two asterisks indicate statistical significance at $p<0.05$ and $p<0.01$ respectively.

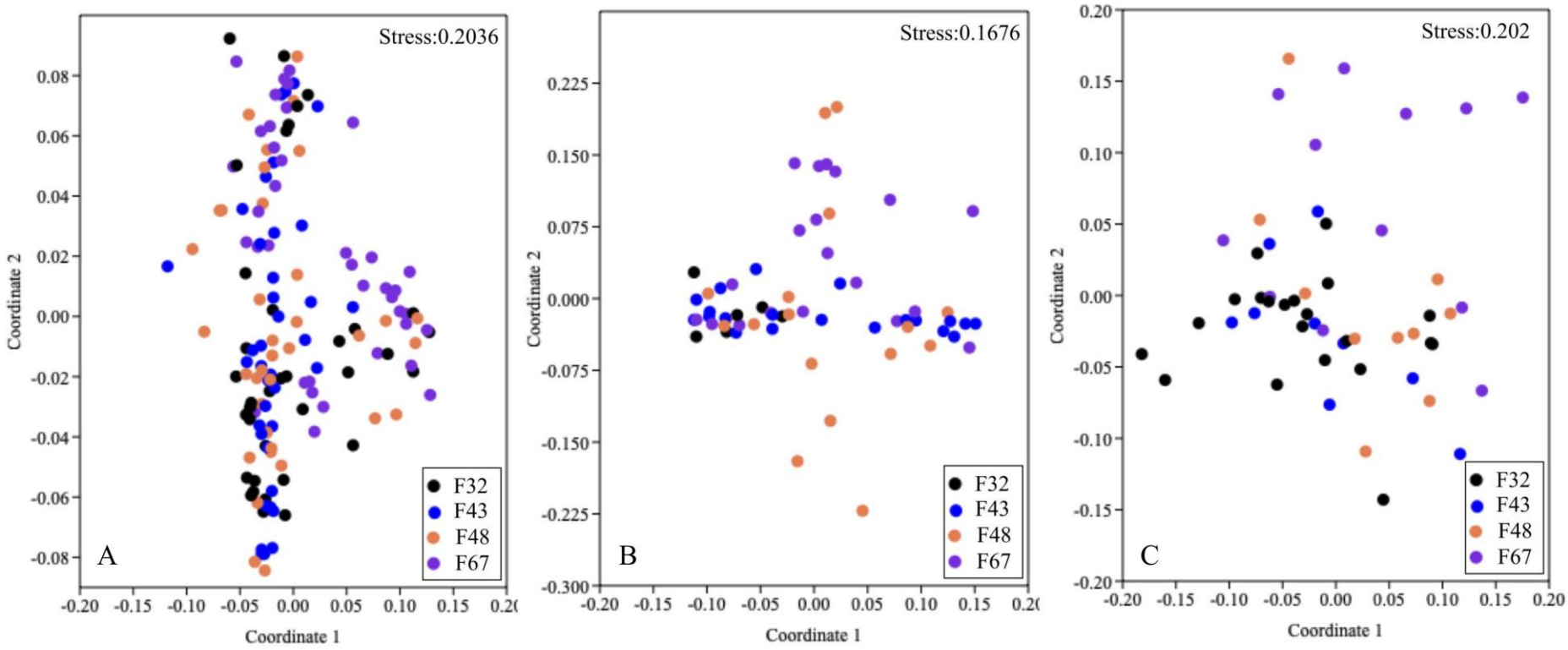


Figure 4-2: 3D nMDS plot based on Bray-Curtis dissimilarity for comparison of the SRO microbiota across families within the pre-QX (A), uninfected (B) and QX-infected groups (C). Axes 1 and 2 are plotted.

Table 4-3: PEMANOVA results comparing the microbiota between families in each group.

Family	F32	F43	F48
Pre-QX			
F43	F = 1.78, p = 0.0059		
F48	F = 1.421, p = 0.0607	F = 1.548, p = 0.0265	
F67	F = 3.696, p = 0.0001	F = 4.4, p = 0.0001	F = 2.759, p = 0.0022
Uninfected			
F43	F = 1.285, p = 0.164		
F48	F = 1.504, p = 0.0452	F = 1.727, p = 0.0204	
F67	F = 1.401, p = 0.0501	F = 1.872, p = 0.0107	F = 1.143, p = 0.229
QX-infected			
F43	F = 1.366, p = 0.0648		
F48	F = 2.3, p = 0.0008	F = 1.481, p = 0.0243	
F67	F = 1.887, p = 0.0041	F = 1.72, p = 0.0032	F = 2.182, p = 0.001

When using data summarised at the genus level, SIMPER analysis showed substantial variability in the microbiota composition between families in each group and was mainly driven by OTUs assigned to the *Mycoplasma, Candidatus Hepatoplasma* and *Arcobacter* genera (Figure 4-3). In the pre-QX group, SIMPER analyses showed that the microbiota of F32 was 77.57% and 85.5% dissimilar to those of F43 and F67 respectively. An OTU assigned to the *Mycoplasma* genus (OTU 11355) was under-represented in F32 compared to F43 driving 18.1% of the dissimilarity (Supplementary Table 4-4) and, an OTU belonging to the *Arcobacter* genus (OTU 17190) was under-represented in comparison to F67 family driving 14.5% of the dissimilarity (Supplementary Table 4-5). The microbiota of F43 were 77.3% and 81.2% dissimilar to F48 and F67 respectively. An OTU assigned to the *Mycoplasma* genus (OTU 11355) was over-represented in F43 compared to F48 and F67 driving 19.2% and 16.4% of the dissimilarity respectively (Supplementary Table 4-6 and 4-7). The microbiota of F48 was found to be 83.8% dissimilar to those of F67 with an OTU assigned to the *Arcobacter* genus (OTU 17190) under-represented in F48 and contributing 17.5% dissimilarity (Supplementary Table 4-8).

Within the uninfected group, SIMPER analyses showed that the microbiota of F32 was 83.6% dissimilar to those of F48 with an OTU assigned to the *Mycoplasma* genus (OTU 11355) over-represented in F32 compared to F48 and driving 23.1% of the dissimilarity (Supplementary Table 4-9). The microbiota of F43 were 85.7% and 84.8% dissimilar to F48 and F67 respectively. An OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 11357) was over-represented in F43 compared to F48 and F67 driving 17.7% and 18.3% of the dissimilarity respectively (Supplementary Table 4-10 and 4-11).

In the QX-infected group, the microbiota of F32 were 81.9% and 83.7% dissimilar to those of F48 and F67 respectively with an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 11357) over-represented in F32 compared to F48 and F67 and driving 18.7% of the dissimilarity for both (Supplementary Table 4-12 and 4-13). The microbiota of F43 were 79.6% and 83.4% dissimilar to F48 and F67 respectively with an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 11357) over-represented in F43 when compared to F48 and F67 driving 17.4% and 17.5% of the dissimilarity respectively (Supplementary Table 4-14 and 4-15). The microbiota of F48 was found to be 85.2% dissimilar to those of F67 with an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 11357) under-represented in F48 and contributing 9.4% of dissimilarity (Supplementary Table 4-16).

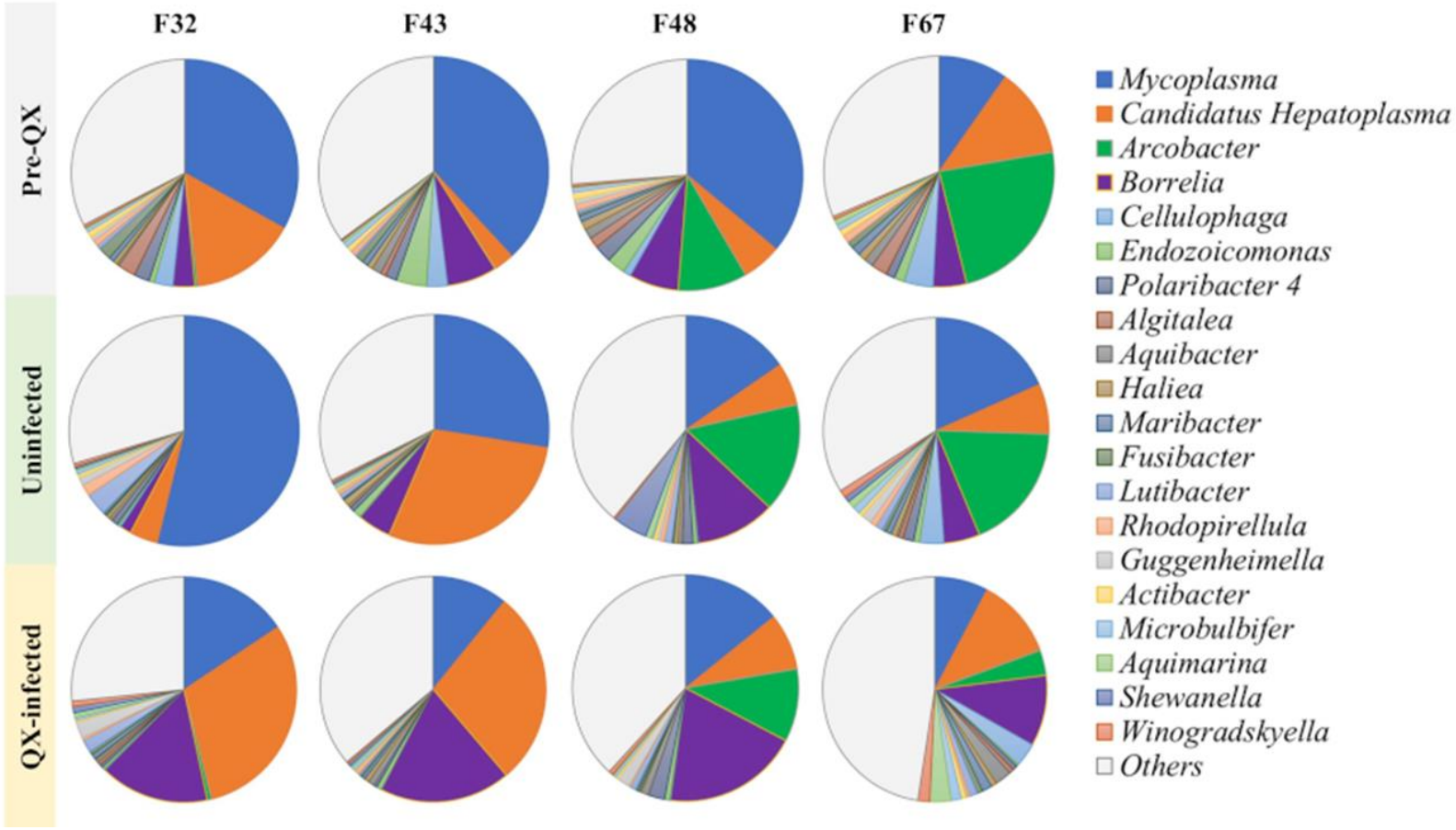


Figure 4-3: Average microbiota composition of SRO showing the top 20 dominant and grouped remaining taxa (others) in families in the pre-QX, uninfected and QX-infected groups. Data is summarised at the genus level.

4.4.3 Core microbiota within and across groups

Given the variability in the SRO microbiota across families, we decided to focus on the core microbiota for each family in the pre-QX, QX-infected and uninfected groups. Each family had a small number of core OTUs ranging from 3 to 15 OTUs (Supplementary Table 4-17). To determine core taxa which consistent occurrence across the families in each group (pre-QX, uninfected and QX-infected), Venn diagrams were generated using the online tool of Bioinformatics & Evolutionary Genomics (Figure 4-4A). An OTU assigned to the *Mycoplasma* genus (OTU 11355) was core only in the pre-QX group whereas, *Candidatus Hepatoplasma* genus (OTU 11357) was core during the QX disease event in the uninfected and QX-infected groups (Figure 4-4A). Notably, OTU 1 from the *Borrelia* genus was core in all groups. We further examined shifts of these core microbiota for each family. Our data showed that the relative abundance of OTU 11355 from the *Mycoplasma* genus was over-represented in pre-QX and uninfected oysters, whereas the relative abundance of OTU 1 from the *Borrelia* genus was more abundant in QX-infected oysters. These shifts were observed in all families (Figure 4-4B). Changes in the relative abundance of OTU 11357 from the *Candidatus Hepatoplasma* genus in pre-QX and QX-infected SROs differed with this member over-represented in QX-infected SROs only for F32 and F48 but not for the other families. Therefore, OTU 11355 and OTU 1 from *Mycoplasma* and *Borrelia* genera respectively were selected for qPCR analyses.

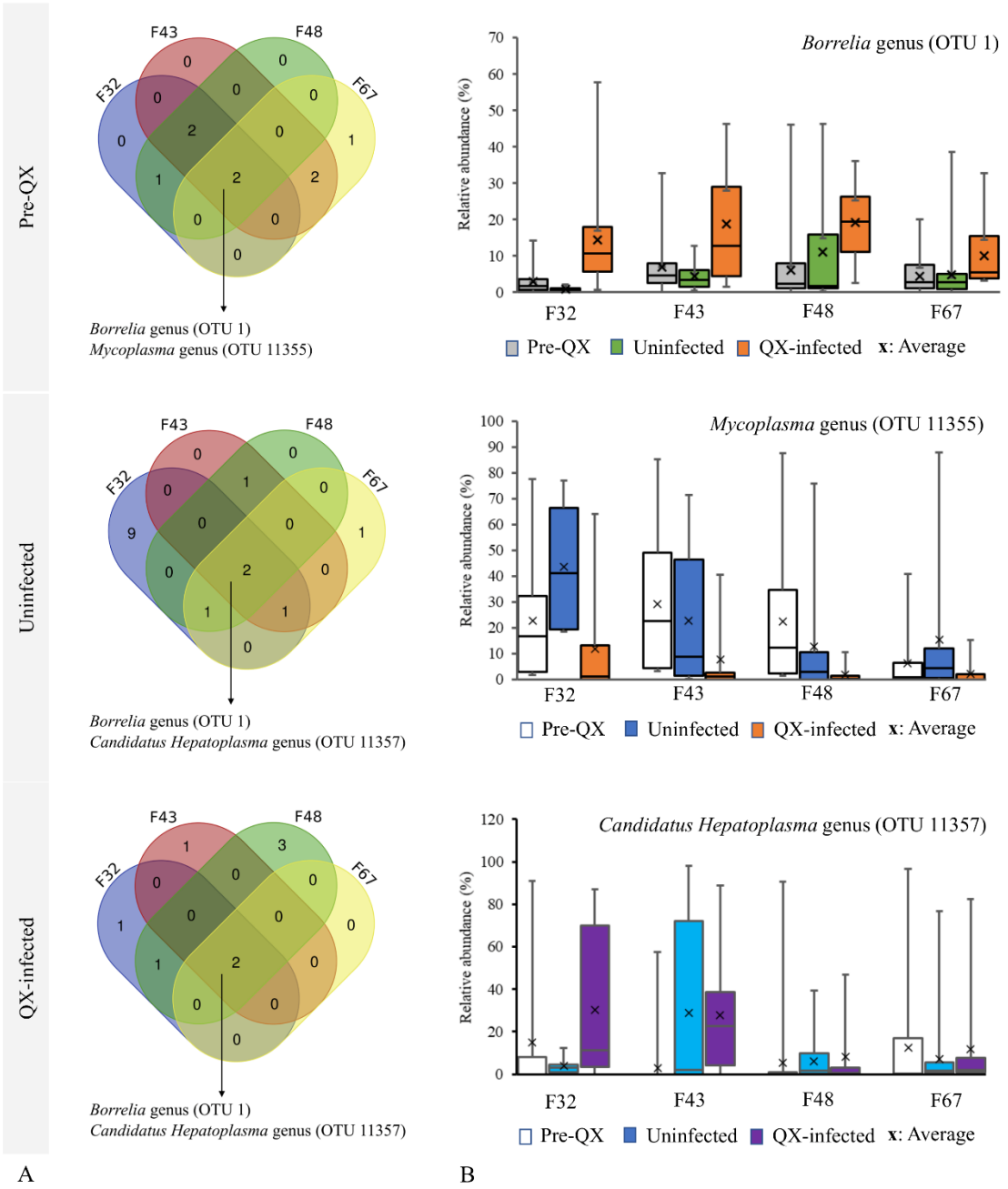


Figure 4-4: Core microbiota and shifts according to health group. Core microbiota is defined as OTUs present in at least 80% of samples. Shared core microbiota between families within pre-QX, uninfected and QX-infected SROs (A). Shifts in average relative abundance of shared core microbiota (B).

4.4.4 Shifts in abundances of *Borrelia* and *Mycoplasma* inferred from qPCR

We performed qPCR to accurately quantify the abundance of *Borrelia* and *Mycoplasma* in the SRO families. Overall, the assay showed the qPCR performance was sufficient to quantify abundance in at least a portion of *Borrelia* and *Mycoplasma* OTUs in our data set. When analysed using the Bio-Rad CFX Maestro 1.1 software, both qPCR assays displayed efficiencies between 96.6% to 98.3% and a single melting curve across all runs. However, this wasn't the case for the *Borrelia* qPCR assay when it was used on the environmental samples with a secondary peak observed between temperatures of 74-76°C in most of the samples and the main product observed around 77-85°C. In order to use this assay on our environmental samples, data was collected only for 15 sec at 77°C for the analyses (Supplementary Figure 4-2).

Overall, the number of copies of *Borrelia* per milligram of adductor tissue was significantly higher compared to *Mycoplasma* (Kruskal-Wallis test, $H = 216$, $p < 0.001$, Figure 4-5). When we compared the abundance of these genera in the QX-infected to both pre-QX and uninfected groups, the number of copies of *Mycoplasma* in QX-infected oysters were significantly higher compared to both pre-QX and uninfected oysters (Kruskal-Wallis test, $H = 26.88$ and 4.195 respectively, $p < 0.05$, Supplementary Table 4-18, Figure 4-6A). The qPCR shift patterns did not correlate with the relative abundance of *Mycoplasma* OTU 11355 in the 16S rRNA gene amplicon sequencing however, relative increases in other *Mycoplasma* including OTU 11599 and OTU 14108 might explain this result (Supplementary Table 4-18, Figure 4-6B). The qPCR shift pattern in all families did not correlate with the relative abundance of *Mycoplasma* OTU 11355 observed in the 16S rRNA gene sequencing data (Supplementary Table 4-19, Figure 4-7).

For the abundance of *Borrelia* genus (OTU 1), the qPCR data displayed the same shift pattern with the 16S rRNA gene sequencing data. The core *Borrelia* genus was significantly more abundant in QX-infected SROs compared to both pre-QX and uninfected SROs (Kruskal-Wallis test, H = 61.2 and 4.16 respectively, $p < 0.05$, Supplementary Table 4-20, Figure 4-8). We then examined the abundance of *Borrelia* in each SRO family. Statistical analyses indicated that shift patterns were consistent between qPCR and 16S rRNA gene sequencing data (Figure 4-9). 16S rRNA gene data showed the relative abundance of *Borrelia* genus (OTU 1) was substantially more abundant in QX-infected SROs compared to both pre-QX and uninfected SROs with all families (Kruskal-Wallis test, $p < 0.05$, Supplementary Table 21, Figure 4-9A). Consistent with this data, qPCR showed the number of copies of *Borrelia* in QX-infected oysters was significantly higher compared to pre-QX in all families and, to uninfected for the F48 and F67 families (Kruskal-Wallis test, $p < 0.05$, Supplementary Table 21, Figure 4-9B)

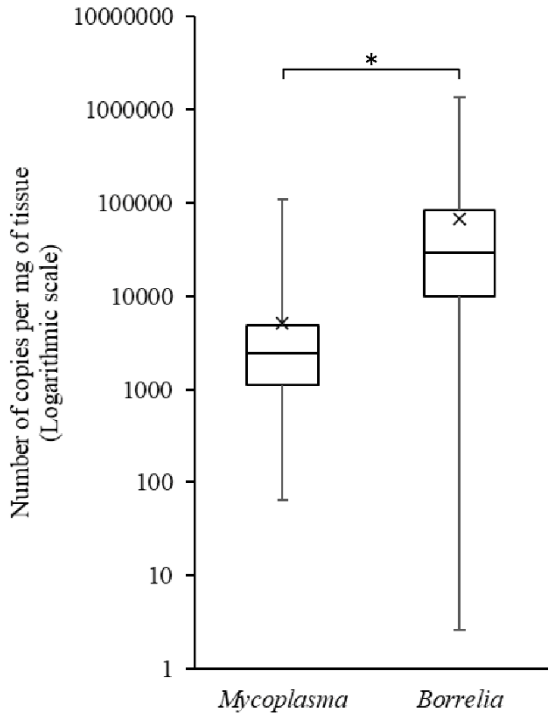


Figure 4-5: Box and whisker plot showing the average number of 16S rRNA gene copies of *Mycoplasma and Borrelia* per mg of adductor tissue from all SROs as determined by qPCR. The x represents the mean of the data set. The asterisk indicates statistical significance at $p < 0.001$.

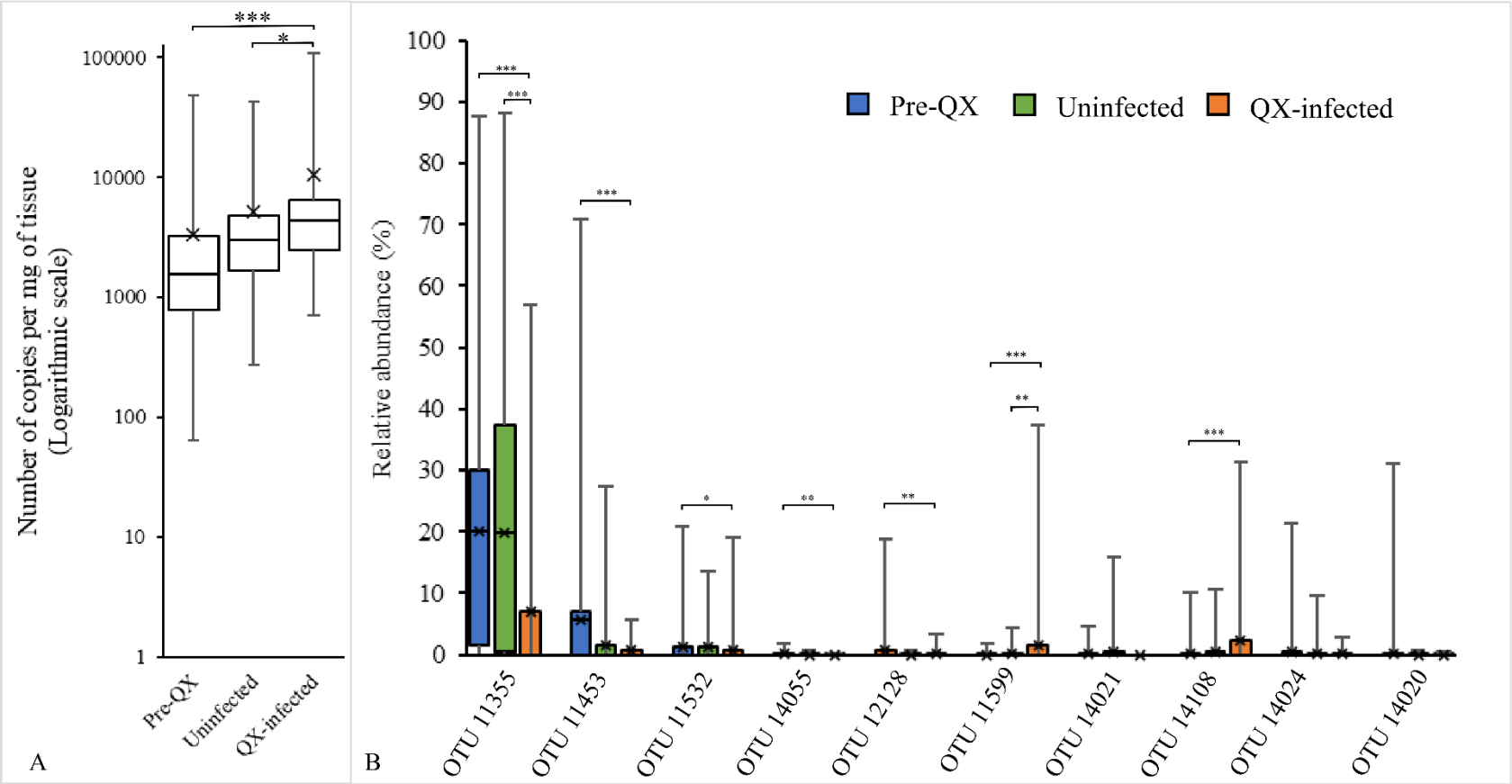


Figure 4-6: Box and whisker plot showing 16S rRNA gene qPCR-measured absolute abundance of *Mycoplasma* groups of SROs (A) and relative abundance of multiple *Mycoplasma* OTUs from 16S rRNA gene sequencing data (B). The x represents the mean of the data set. Single, double, and triple asterisks indicate statistical significance at $p < 0.05$, < 0.01 and < 0.001 respectively.

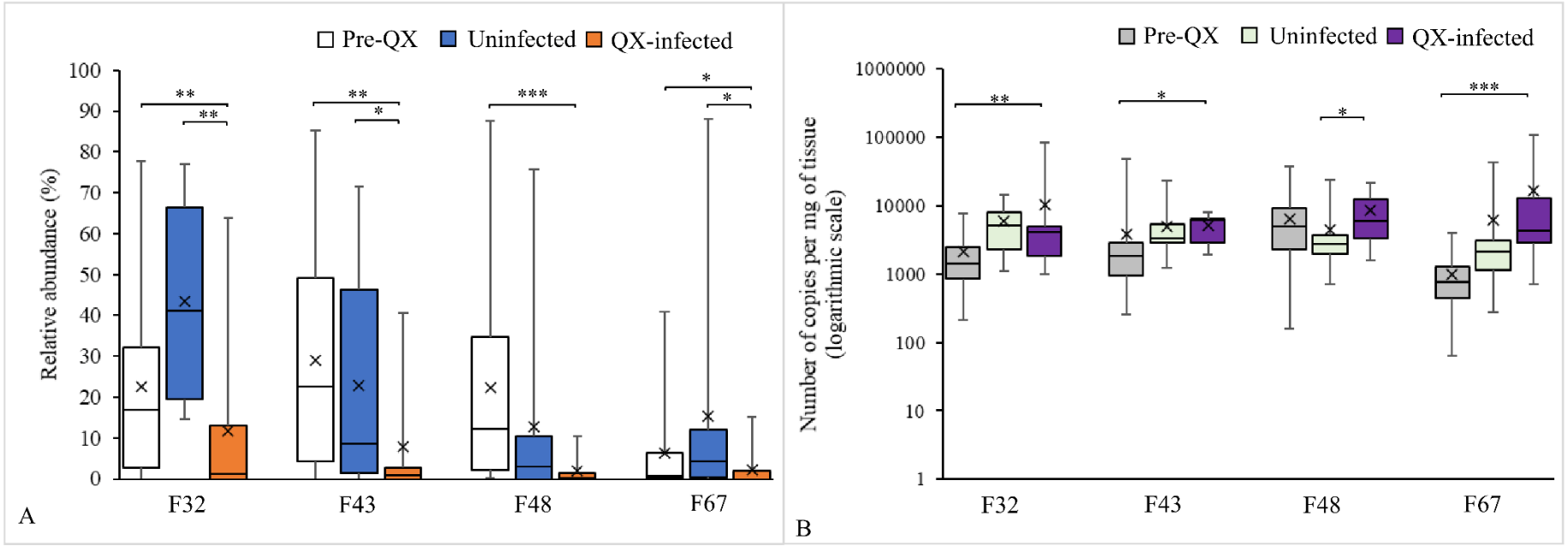


Figure 4-7: Box and whisker plot showing relative abundance of *Mycoplasma* genus (OTU 11355) from 16S rRNA gene sequencing data (A) and 16S rRNA gene qPCR-measured absolute abundance of *Mycoplasma* in groups of SROs for each family (B). The x represents the mean of the data set. The single, double and three asterisks indicate statistical significance at $p < 0.05$, < 0.01 and < 0.001 respectively.

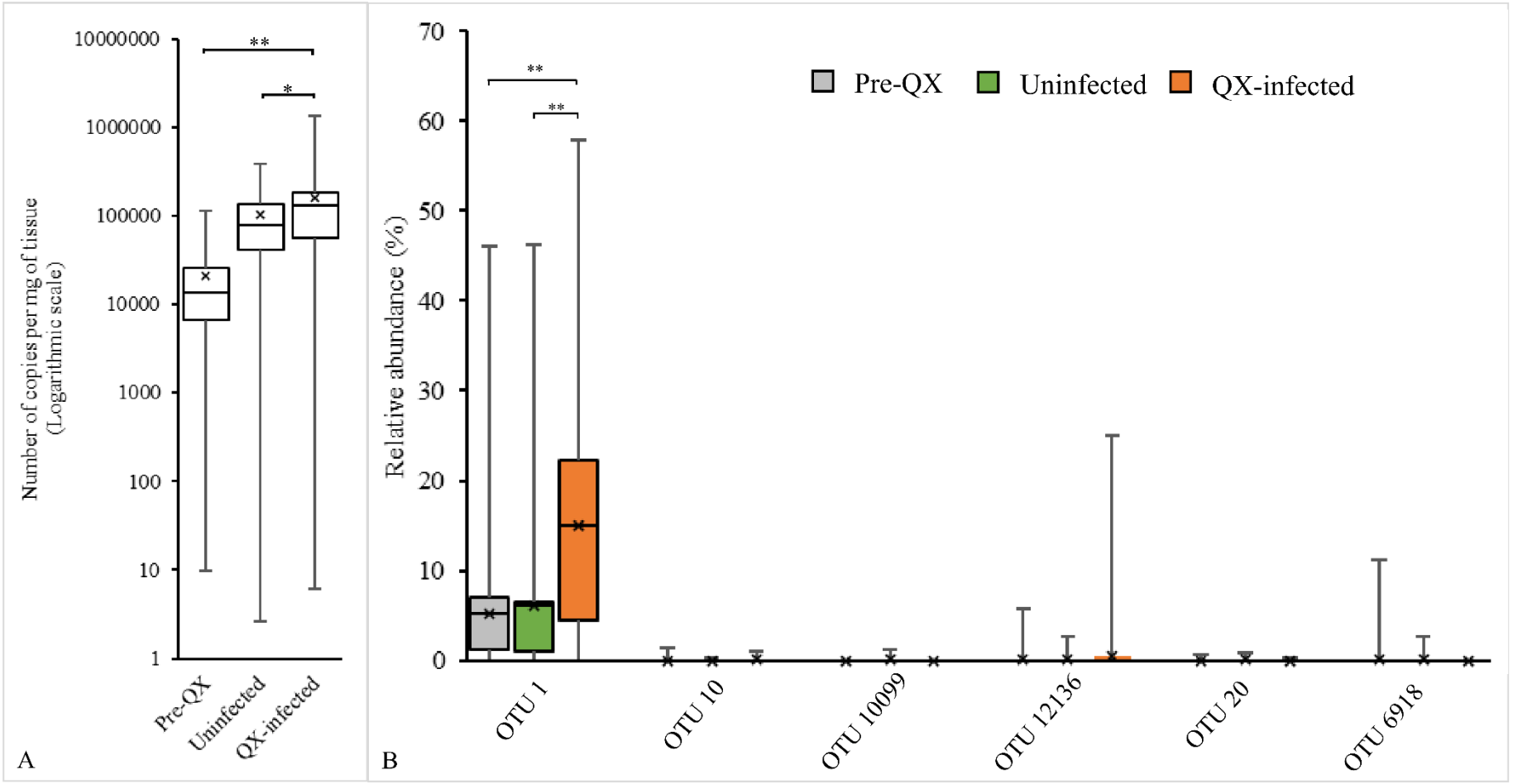


Figure 4-8: Box and whisker plot showing 16S rRNA gene qPCR-measured absolute abundance of *Borrelia* in groups of SROs (A) and relative abundance of multiple *Borrelia* OTUs from 16S rRNA gene sequencing data (B). The x represents the mean of the data set. The single and double asterisks indicate statistical significance at $p < 0.05$ and < 0.001 respectively.

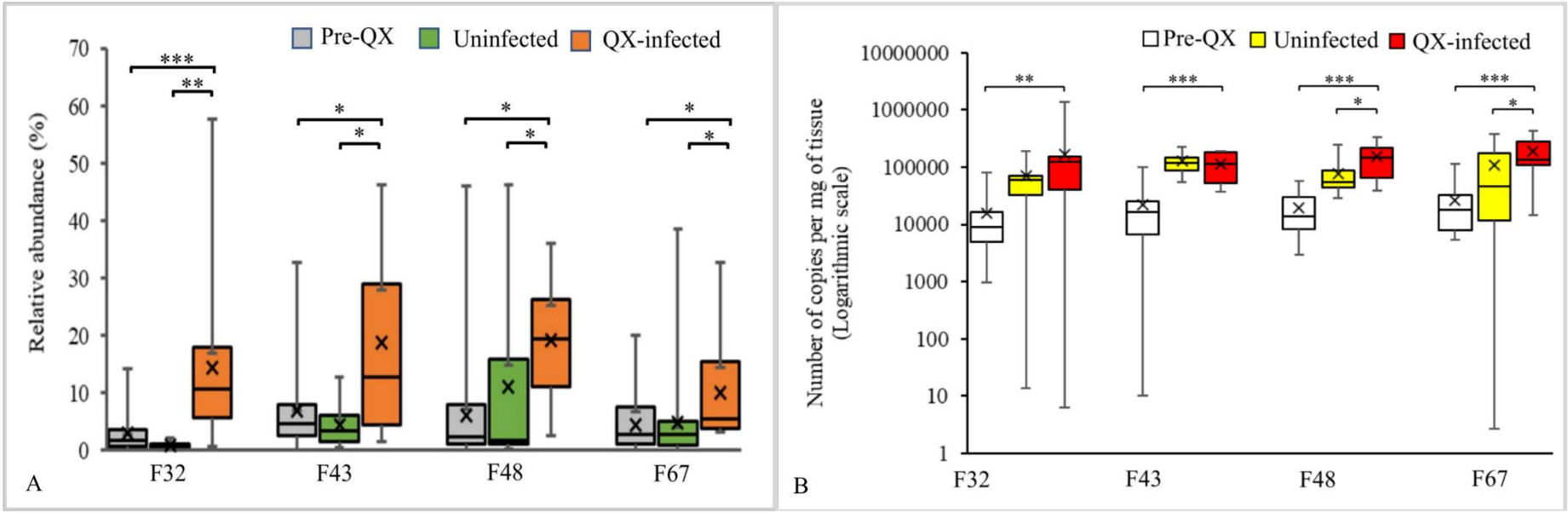


Figure 4-9: Box and whisker plot showing relative abundance of *Borrelia* genus (OTU 1) from 16S rRNA gene sequencing data (A) and 16S rRNA gene qPCR-measured absolute abundance of *Borrelia* in groups of SROs for each family (B). The x represents the mean of the data set. The single, double and three asterisks indicate statistical significance at $p < 0.05$, < 0.01 and < 0.001 respectively.

4.5 Discussion

The principal goal of this study was to investigate the SRO microbiota between four SRO families with intermediate QX resistance and then to identify a core microbiota relating to healthy and QX-infected oysters. Although the familes are genetically distinct, they all showed a similar phenotype with regard to resistance to QX disease. In chapter 2, we showed an influence of genetics on the oyster microbiota (King *et al.*, 2019c; Nguyen *et al.*, 2020; Wegner *et al.*, 2013) with those from SROs resistant to QX having a different structure to susceptible SROs however, this effect was observed only in winter (Nguyen *et al.*, 2020). In chapter 3, we observed that when the families were grouped according to health or disease state, distinct differences in microbiota structure were observed. However, when families were looked at in isolation, substantial variation was observed and were principally characterised by differences in the relative abundance of an OTU assigned to *Candidatus Hepatoplasma* genus. Although the role of *Candidatus Hepatoplasma* genus within SROs is unknown, we have previously observed that the relative abundance of this genus in QX-sensitive oysters is higher when compared to those in QX-resistant oysters (Chapter 2; (Nguyen *et al.*, 2020)) suggesting that this bacterium may have an important role in QX disease or disease susceptibility. If this is indeed the case, its role may be dependent on the genetics of the SRO.

To identify microbiota trends that were consistent across families, we decided to investigate the dynamics of core OTUs (Yao *et al.*, 2018). Despite the significant heterogeneity in the oyster microbiota across families, we successfully identified a core microbiota for each family. Notably, an OTU belonging to *Mycoplasma* was identified as core across all families in the pre-QX group. Additionally, an OTU belonging to the *Candidatus Hepatoplasma* was core across the families in the uninfected and QX-infected groups and an OTU from the *Borrelia*

genera was core across all families in all groups. Interestingly, *Borrelia* belongs to the Spirochaete phylum (Margos *et al.*, 2020) with sequences from this phylum also identified as core in the Pacific oyster across 6 estuaries in NSW, Australia spanning a distance of approximately 470 kilometres (King *et al.*, 2020). Of the core OTUs, the relative abundance shifts were consistent in all families for *Mycoplasma* and *Borrelia* and so these were selected for the design of qPCR assays to investigate their absolute abundance in the SRO samples.

Contrary to our expectations, qPCR data indicated that *Mycoplasma* in QX-infected SROs was significantly higher when compared to SROs from the pre-QX and uninfected groups. This might be explained by the non-specificity of the qPCR primers which would likely amplify more *Mycoplasma* OTUs than the one that we targeted. In the SRO microbiota, we observed an increased relative abundance of two other *Mycoplasma* OTUs which accounted for a small proportion in the SRO microbiota. Combined with our qPCR data, this may indicate a replacement of one type of *Mycoplasma* OTU with others as been observed in Pacific oysters, where summer mortality is preceded by a progressive replacement of a benign *Vibrio* population in the microbiota with a virulent population (Lemire *et al.*, 2015). Although the role of these *Mycoplasma* OTUs with QX disease in SROs is unknown, it is the case that *Mycoplasma* have previously been considered as potential pathogens in bivalves (Paillard *et al.*, 2004). Future studies are required to investigate this idea and could be accomplished by sequencing the *Mycoplasma* PCR amplicon or determining whether there is a shift in the *Mycoplasma* population using techniques such as denaturing gradient gel electrophoresis (DGGE) (Strathdee & Free, 2013).

Consistent with 16S rRNA gene sequencing data, our qPCR data showed the number of *Borrelia* in QX-infected oysters was significantly higher when compared to both pre-QX and

uninfected oysters and may indicate that the abundance of *Borrelia* in SROs could be used for assessing the health of SROs. However, when we examined changes in the abundance of *Borrelia* for each family, the shift pattern was not consistent with two families (F32 and F43) showing no statistical difference between QX-infected and uninfected SROs during the outbreak. Further optimisation of the *Borrelia* qPCR such as designing and testing other primer sets that eliminate the secondary product or increasing specificity by converting this assay to a probe assay (Arya *et al.*, 2005) might improve its utility.

4.6 Conclusion

The SRO microbiota displays variation across families however, two core OTUs belonging to the *Mycoplasma* and *Borrelia* genera were found to have similar patterns in relative abundance across all families. qPCR and 16S rRNA gene data indicate a possible replacement of one type of *Mycoplasma* with another either just before or during QX disease. Overall, our qPCR data of the *Borrelia* genera was consistent with the 16S rRNA gene data however, further research and development of the qPCR assay is required to better determine its use as an indicator of SRO health and/or sensitivity to QX disease.

4.7 Supplementary information

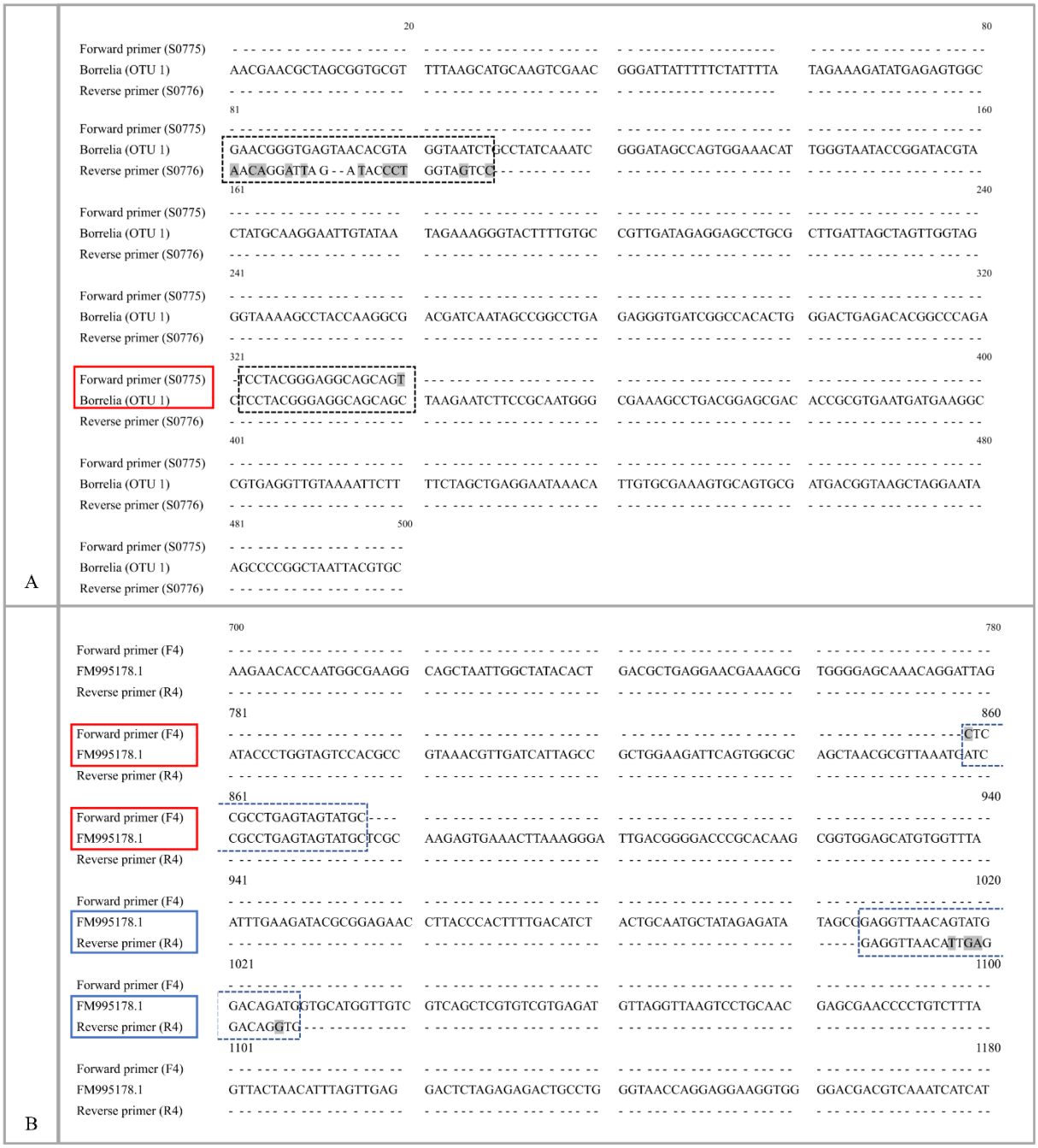
Supplementary Table 4-1: The sequences of an OTU assigned to *Borrelia* genus (OTU 1) and *Mycoplasma* genus (OTU 11355).

OTU	Sequence
OTU 1	AACGAACGCTAGCGGTGCGTTTTAAGCATGCAAGTCGAACGGGATTA TTTTTCTATTTTATAGAAAGATATGAGAGTGGCGAACGGGTGAGTAAC ACGTAGGTAATCTGCCTATCAAATCGGGATAGCCAGTGGAAACATTG GGTAATACCGGATACGTACTATGCAAGGAATTGTATAATAGAAAGGG TACTTTTGTGCCGTTGATAGAGGAGCCTGCGCTTGATTAGCTAGTTGG TAGGGTAAAAGCCTACCAAGGCGACGATCAATAGCCGGCCTGAGAGG GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCAGCTAAGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCG ACACCGCGTGAATGATGAAGGCCGTGAGGTTGTAAAATTCTTTTCTAG CTGAGGAATAAACATTGTGCGAAAGTGCAGTGCGATGACGGTAAGCT AGGAATAAGCCCCGGCTAATTACGTGC
OTU 11355	GATGAACGCTGGCTGTGTGCCTAATACATGCATGTCGAACGAAGCAC TTGTGCTTAGTGGCGAATGGGTGAGTAACACGTACTTAATCTGCCCTC AAGTCTGGGACAACAGTTGGAAACGACTGCTAATACCGGATATGTAT TCTTATCGCATGATAAGTTTATAAAAGGAGCGTTTGCTTCGCTAGAGG ATGAGAGTGCGCCATATTAGCTAGTTGGTAGGGTAAGAGCCTACCAA GGCGATGATATGTAGCGGGGTCGAGAGGCTGAACCGCCACACTGGGA CTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATT CCACAATGAACGAAAGTTTGATGGAGCGACACAGCGTGGAGGATGAA GGTTTTCGGATCGTAAACTCCTGTTATAAGAGAAGAACAAGCTTAAT AGGAAATGATTAAGCCCTGACGGTACCTTATCAGAAAGCACCGGCA AACTATGTGCCAG

Supplementary Table 4-2: Previously published primers for *Mycoplasma* and *Borrelia* species amplification.

Bacteria	Primer	Nucleotide sequence (5' – 3')	Amplicon size	Reference
Borrelia	nTM17.F	GTGGATCTATTGTATTAGATGAGGCTCTCG	222 bp	(Morrison *et al.*, 1999)
	nTM17.R	GCCAAAGTTCTGCAACATTAACACCTAAAG		
	p16Swt-fwd	GGATATAGTTAGAGATAATTATTCCCCGTTTG	139 bp	(O'Rourke *et al.*, 2013)
	p16Swt-rev	CATTACATGCTGGTAACAGATAACAAGG		
	Bor-16S-F	GGTCAAGACTGACGCTGAGTCA	136 bp	(De Leeuw *et al.*, 2014)
	Bor-16S-F	GGCGGCCACTTAACACGTTAG		
	S0775	TCCTACGGGAGGCAGCAGT	466 bp*	(Nadkarni *et al.*, 2002; Panetta *et al.*, 2017)
	S0776	GGACTACCAGGGTATCTAATCCTGTT		
Mycoplasma	1F	GGATTAGATACCCTAGTAGTCCACA	186 bp**	(Salling & Bang-Christensen, 2016)
	1R	CGTGTACCGTCGAATTAAGCA		
	2F	ACTAAGTGTTGGCCAAAAGGTC	199 bp***	
	2R	CCTCCGAATTTATTTCTAAGCCTTTG		
	3R	TCATCATGCCTCTTACGAGTG	204 bp****	
	3F	GCGGTGTGTACAAGACCCGA		
	4F	CTCCGCCTGAGTAGTATGC	169 bp*****	
	4R	CACCTGTCTCAATGTTAACCTC		

*: universal primers **: *Mycoplasma alvi*, ***: *Acholeplasma laidlawii*, ****: *M .adleri,* *****: *M. capricolum subsp. capricolum*



Supplementary Figure 4-1: Alignment of primers (S0775/S0776) to *Borrelia* genus (OTU 1) sequence (A). Alignment of *Mycoplasma* primers 4 (F4/R4) to the 16S rRNA gene nucleotide sequence of FM995178.1 with 99.79% identity to the partial 16S rRNA gene sequence of *Mycoplasma* OTU 11355 (B).

Supplementary Table 4-3: Kruskal-Wallis results of the alpha diversity indices of each family compared to others in the pre-QX, uninfected and QX-infected groups. Alpha indices include species richness (Chao1) species evenness (Simpson) and species diversity (Shannon).

Comparison	Species richness		Species evenness		Species diversity	
	H	p value	H	p value	H	p value
Pre-QX						
F32 vs F43	0.1516	0.697	0.2475	0.6189	0.6063	0.4362
F32 vs F48	0.1466	0.7018	0.02854	0.8658	0.008118	0.9282
F32 vs F67	1.302	0.2539	0.0004678	0.9827	0.007485	0.9311
F43 vs F48	0.007486	0.9311	0.2584	0.6112	0.4211	0.5164
F43 vs F67	1.342	0.2467	0.04317	0.8354	0.1399	0.7084
F48 vs F67	1.493	0.2217	0.09836	0.7538	0.009474	0.9225
Uninfected						
F32 vs F43	0.003704	0.9515	0.3	0.5839	0.1333	0.715
F32 vs F48	0.1225	0.7263	0.3137	0.5754	0.3971	0.5286
F32 vs F67	0.03333	0.8551	0.05926	0.8077	0.1333	0.715
F43 vs F48	0.2232	0.6366	0.008359	0.9272	0.3353	0.5626
F43 vs F67	0.002927	0.9569	1.171	0.2793	0.7032	0.4017
F48 vs F67	0.5944	0.8074	2.322	0.1276	2.322	0.1276
QX-infected						
F32 vs F43	2.299	0.1295	0.32	0.5716	1.037	0.3086
F32 vs F48	0.4571	0.499	2.187	0.1391	3.002	0.08317
F32 vs F67	1.767	0.1838	5.25	0.02498	6.961	0.0083
F43 vs F48	1.127	0.2885	0.54	0.4624	0.54	0.4624
F43 vs F67	0.2079	0.6484	2.426	0.1194	1.571	0.21
F48 vs F67	0.1269	0.2599	1.433	0.2313	1.607	0.205

Supplementary Table 4-4: SIMPER analysis of the SRO microbiota between F32 and F43 within pre-QX group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F32	Mean F43
Mycoplasma genus (OTU 11355)	18.08	22.6	29.1
Candidatus Hepatoplasma genus (OTU 11357)	11.07	15.1	2.81
*Mycoplasm*a genus (OTU 11453)	6.012	6.02	5.7
Borrelia genus (OTU 1)	3.967	2.93	7.01
Cellulophaga genus (OTU 9296)	3.125	2.62	2.89
Family XIII family (OTU 11608)	2.473	1.52	2.81
Flavobacteriaceae family (OTU 4366)	2.297	1.66	2.05
Mycoplasma genus (OTU 11532)	1.945	1.68	2.01
Algitalea genus (OTU 8064)	1.875	2.56	0.683
Mycoplasmataceae family (OTU 375)	1.733	1.94	1.55

Supplementary Table 4-5: SIMPER analysis of the SRO microbiota between F32 and F67 within pre-QX group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F32	Mean F67
Arcobacter genus (OTU 17190)	14.47	0.351	23.8
Candidatus Hepatoplasma genus (OTU 11357)	13.56	15.1	12.6
Mycoplasma genus (OTU 11355)	12.91	22.6	6.38
Mycoplasma genus (OTU 11453)	4.006	6.02	2.22
Cellulophaga genus (OTU 9296)	3.238	2.62	4.02
Borrelia genus (OTU 1)	2.603	2.93	4.57
Flavobacteriaceae family (OTU 4366)	2.432	1.66	2.74
Algitalea genus (OTU 8064)	2.384	2.56	2.2
Mycoplasmataceae family (OTU 375)	1.895	1.94	2.07
Polaribacter 4 genus (OTU 9546)	1.277	2.15	1.08

Supplementary Table 4-6: SIMPER analysis of the SRO microbiota between F43 and F48 within pre-QX group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F48
Mycoplasma genus (OTU 11355)	19.16	29.1	22.3
Mycoplasma genus (OTU 11453)	7.224	5.7	8.79
Arcobacter genus (OTU 17190)	6.455	0.0741	9.67
Candidatus Hepatoplasma genus (OTU 11357)	5.204	2.81	5.52
Borrelia genus (OTU 1)	4.842	7.01	6.11
Cellulophaga genus (OTU 9296)	2.325	2.89	1.08
*Endozoicomona*s genus (OTU 2823)	2.299	2.3	2.33
Family XIII family (OTU 11608)	2.161	2.81	0.914
Polaribacter 4 genus (OTU 9546)	1.768	1.59	2.32
Mycoplasma genus (OTU 11532)	1.705	2.01	1.08

Supplementary Table 4-7: SIMPER analysis of the SRO microbiota between F43 and F67 within pre-QX group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F67
Mycoplasma genus (OTU 11355)	16.38	29.1	6.38
Arcobacter genus (OTU 17190)	14.45	0.0741	23.8
Candidatus Hepatoplasma genus (OTU 11357)	8.448	2.81	12.6
Mycoplasma genus (OTU 11453)	3.869	5.7	2.22
Borrelia genus (OTU 1)	3.748	7.01	4.57
Cellulophaga genus (OTU 9296)	3.345	2.89	4.02
Flavobacteriaceae family (OTU 4366)	2.562	2.05	2.74
Family XIII family (OTU 11608)	1.776	2.81	0.247
Mycoplasmataceae family (OTU 375)	1.723	1.55	2.07
Endozoicomonas genus (OTU 2823)	1.575	2.3	1.22

Supplementary Table 4-8: SIMPER analysis of the SRO microbiota between F48 and F67 within pre-QX group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F48	Mean F67
Arcobacter genus (OTU 17190)	17.46	9.67	23.8
Mycoplasma genus (OTU 11355)	13.39	22.3	6.38
Candidatus Hepatoplasma genus (OTU 11357)	9.843	5.52	12.6
Mycoplasma genus (OTU 11453)	5.477	8.79	2.22
Borrelia genus (OTU 1)	3.797	6.11	4.57
Cellulophaga genus (OTU 9296)	2.603	1.08	4.02
Flavobacteriaceae family (OTU 4366)	1.928	0.642	2.74
Algitalea genus (OTU 8064)	1.897	1.48	2.2
Mycoplasmataceae family (OTU 375)	1.702	1.21	2.07
Endozoicomonas genus (OTU 2823)	1.652	2.33	1.22

Supplementary Table 4-9: SIMPER analysis of the SRO microbiota between F32 and F48 within uninfected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F32	Mean F48
Mycoplasma genus (OTU 11355)	23.14	43.5	12.7
Arcobacter genus (OTU 17190)	9.559	0	15.4
Borrelia genus (OTU 1)	6.467	0.921	11.2
Flavobacteriaceae family (OTU 11123)	5.691	1.25	8.6
Candidatus Hepatoplasma genus (OTU 11357)	4.132	3.95	6.07
Mycoplasma genus (OTU 11453)	3.456	5.2	0.916
Shewanella genus (OTU 1149)	3.186	0.31	4.91
Flexithrix genus (OTU 9477)	1.704	0	2.75
Planctomycetaceae family (OTU 15557)	1.419	2.02	1.53
Mycoplasma genus (OTU 11532)	1.309	1.74	0.647

Supplementary Table 4-10: SIMPER analysis of the SRO microbiota between F43 and F48 within uninfected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F48
Candidatus Hepatoplasma genus (OTU 11357)	17.67	28.9	6.07
Mycoplasma genus (OTU 11355)	14.91	22.7	12.7
Arcobacter genus (OTU 17190)	9.282	0.0219	15.4
*Borreli*a genus (OTU 1)	6.227	4.44	11.2
Flavobacteriaceae family (OTU 11123)	5.328	0.521	8.6
Shewanella genus (OTU 1149)	3.079	0.298	4.91
Mycoplasmataceae family (OTU 375)	1.698	1.89	1.29
Flexithrix genus (OTU 9477)	1.69	0.0924	2.75
Mycoplasma genus (OTU 11532)	1.689	2.48	0.647
Planctomycetaceae family (OTU 15557)	1.212	1.24	1.53

Supplementary Table 4-11: SIMPER analysis of the SRO microbiota between F43 and F67 within uninfected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F67
Candidatus Hepatoplasma genus (OTU 11357)	18.3	28.9	7.19
Mycoplasma genus (OTU 11355)	16.03	22.7	15.4
Arcobacter genus (OTU 17190)	11.21	0.0219	18.2
Borrelia genus (OTU 1)	3.08	4.44	4.9
Cellulophaga genus (OTU 9296)	2.074	0.0595	3.42
Mycoplasmataceae family (OTU 375)	1.975	1.89	1.88
Mycoplasma genus (OTU 11532)	1.513	2.48	0.093
Mycoplasma genus (OTU 11453)	1.073	0.828	1.31
Guggenheimella genus (OTU 14367)	1.028	0.185	1.56
Polaribacter 4 genus (OTU 9546)	0.9497	0.626	1.56

Supplementary Table 4-12: SIMPER analysis of the SRO microbiota between F32 and F48 within QX-infected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F32	Mean F48
Candidatus Hepatoplasma genus (OTU 11357)	18.65	30.4	8.06
Borrelia genus (OTU 1)	9.105	14.4	19.2
Mycoplasma genus (OTU 11355)	7.595	11.8	1.82
Arcobacter genus (OTU 17190)	6.674	0.562	10.4
Mycoplasma genus (OTU 14108)	4.962	0.624	7.97
Flavobacteriaceae family (OTU 11123)	3.59	0.704	5.57
Lentimicrobiaceae family (OTU 12134)	2.446	1.04	3.8
Guggenheimella genus (OTU 14367)	2.014	2.37	1.86
Desulfobulbaceae family (OTU 2532)	1.34	2.18	0
Polaribacter 4 genus (OTU 9546)	1.312	0.424	2.33

Supplementary Table 4-13: SIMPER analysis of the SRO microbiota between F32 and F67 within QX-infected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F32	Mean F67
Candidatus Hepatoplasma genus (OTU 11357)	18.7	30.4	11.7
Mycoplasma genus (OTU 11355)	7.635	11.8	2.25
Borrelia genus (OTU 1)	7.41	14.4	10.1
Mycoplasma genus (OTU_11599)	2.569	0.628	3.8
Arcobacter genus (OTU 17190)	2.421	0.562	3.63
Pricia genus (OTU_5718)	2.22	0.788	3.36
Cellulophaga genus (OTU 9296)	2.125	0.129	3.36
Prolixibacter genus (OTU 4662)	1.72	0.727	2.3
Flammeovirgaceae family (OTU 10676)	1.627	0.192	2.6
Guggenheimella genus (OTU 14367)	1.48	2.37	0.176

Supplementary Table 4-14: SIMPER analysis of the SRO microbiota between F43 and F48 within QX-infected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F48
Candidatus Hepatoplasma genus (OTU 11357)	17.36	27.9	8.06
Borrelia genus (OTU 1)	10.1	18.8	19.2
Arcobacter genus (OTU 17190)	6.753	0	10.4
Mycoplasma genus (OTU 11355)	5.348	7.75	1.82
Mycoplasma genus (OTU 14108)	5.079	0.293	7.97
Candidatus Endoecteinascidia genus (OTU 10028)	4.483	6.87	0.144
Flavobacteriaceae family (OTU 11123)	3.717	1.12	5.57
Lentimicrobiaceae family (OTU 12134)	2.53	1.02	3.8
Polaribacter 4 genus (OTU 9546)	1.38	0.432	2.33
Desulfuromusa genus (OTU 15907)	1.279	0.507	1.71

Supplementary Table 4-15: SIMPER analysis of the SRO microbiota between F43 and F67 within QX-infected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F43	Mean F67
Candidatus Hepatoplasma genus (OTU 11357)	17.54	27.9	11.7
Borrelia genus (OTU 1)	9.339	18.8	10.1
Mycoplasma genus (OTU 11355)	5.333	7.75	2.25
Candidatus Endoecteinascidia genus (OTU 10028)	4.324	6.87	0.307
Mycoplasma genus (OTU 11599)	3.047	1.52	3.8
Pricia genus (OTU 5718)	2.228	0.553	3.36
Arcobacter genus (OTU 17190)	2.225	0	3.63
Cellulophaga genus (OTU 9296)	2.115	0.0451	3.36
Prolixibacter genus (OTU 4662)	1.902	1.27	2.3
Flammeovirgaceae family (OTU 10676)	1.597	0	2.6

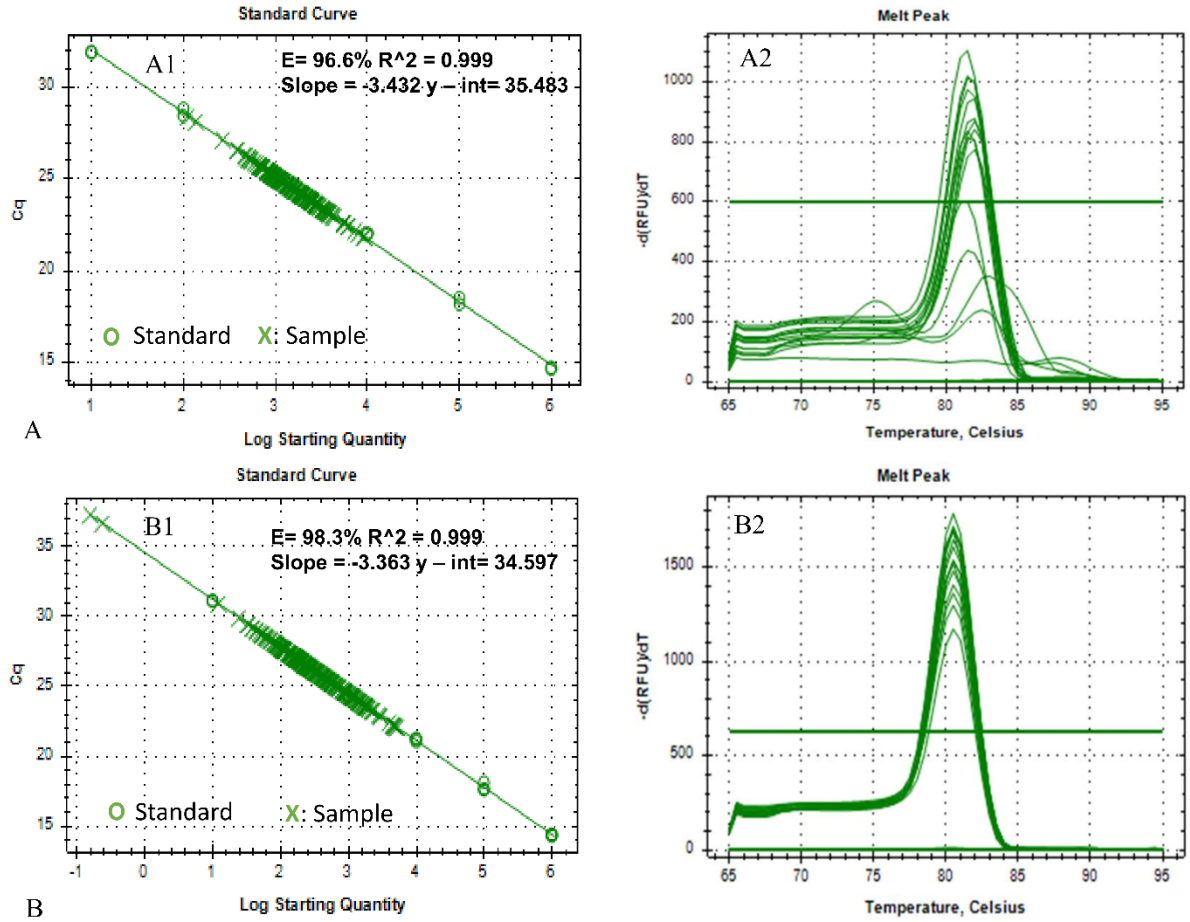
Supplementary Table 4-16: SIMPER analysis of the SRO microbiota between F48 and F67 within QX-infected group. The top 10 OTUs are displayed with their dissimilarity contribution and mean representation. Dissimilarity contribution is cumulative.

Taxon	Contrib. %	Mean F48	Mean F67
Candidatus Hepatoplasma genus (OTU 11357)	9.351	8.06	11.7
Borrelia genus (OTU 1)	8.199	19.2	10.1
Arcobacter genus (OTU 17190)	7.483	10.4	3.63
Mycoplasma genus (OTU 14108)	5.001	7.97	1.4
Flavobacteriaceae family (OTU 11123)	3.356	5.57	0.29
Mycoplasma genus (OTU 11599)	2.586	0.721	3.8
Lentimicrobiaceae family (OTU 12134)	2.273	3.8	0
Cellulophaga genus (OTU 9296)	2.063	0.0968	3.36
Pricia genus (OTU 5718)	2.01	0.146	3.36
Mycoplasma genus (OTU 11355)	1.947	1.82	2.25

Supplementary Table 4-17: Core microbiotas identified in four SRO families in the pre-QX, uninfected and QX-infected groups. Core microbiota was defined as an OTU present in at least 80% of samples.

Family line	Pre-QX	Uninfected	QX-infected
F32	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)
	Mycoplasma genus (OTU 11355)	*DEV007* family (OTU 113)	*Mycoplasma* genus (OTU 11355)
	Comamonadaceae family (OTU 14443)	*Mycoplasma* genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)
	Polaribacter 4 genus (OTU 9546)	*Candidatus Hepatoplasma* genus (OTU 11357)	*Polaribacter 4* genus (OTU 9546)
	Flavobacteriaceae family (OTU 9833)	*Haliea* genus (OTU 12961)	
		Planctomycetaceae family (OTU 15557)	
		Rhodopirellula genus (OTU 15867)	
		H2-104-2 family (OTU 3233)	
		Mycoplasmataceae family (OTU 375)	
		JTB255 marine benthic group family (OTU 6615)	
		Marinifilum genus (OTU 8076)	
		Aureispira genus (OTU 9355)	
		Flavobacteriaceae family (OTU 9833)	
F43	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)
	DEV007 family (OTU 113)	*Mycoplasma* genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)
	Mycoplasma genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)	*JTB255 marine benthic group* family (OTU 5026)
	Mycoplasmataceae family (OTU 375)	*Polaribacter 4* genus (OTU 9546)	
	Polaribacter 4 genus (OTU 9546)		
	Flavobacteriaceae family (OTU 9833)		

F48	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)
	Mycoplasma genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)	*Candidatus Hepatoplasma* genus (OTU 11357)
	Comamonadaceae family (OTU 14443)	*Polaribacter 4* genus (OTU 9546)	*Desulfovibrio* genus (OTU 5030)
	Polaribacter 4 genus (OTU 9546)	*Flavobacteriaceae* family (OTU 9833)	*Marinifilum* genus (OTU 8076)
	Flavobacteriaceae family (OTU 9833)		*Polaribacter 4* genus (OTU 9546)
			Flavobacteriaceae family (OTU 9833)
F67	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)	*Borrelia* genus (OTU 1)
	DEV007 family (OTU 113)	*Mycoplasma* genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)
	Mycoplasma genus (OTU 11355)	*Candidatus Hepatoplasma* genus (OTU 11357)	
	Mycoplasmataceae family (OTU 375)	*Arcobacter* genus (OTU 17190)	
	Cellulophaga genus (OTU 9296)	*Flavobacteriaceae* family (OTU 9833)	



Supplementary Figure 4-2: Performance of *Borrelia* primers (A) and *Mycoplasma* primers (B). Plot of standard curve with linear regression trend line and correlation coefficient for *Borrelia* (A3) and *Mycoplasma* (B3). The correlation coefficients R^2 of standard curves for *Borrelia* and *Mycoplasma* assays displayed acceptable results (99.9%). Melting curves of standard for *Borrelia* (A2) showing a secondary peak between temperatures of 74-76^{0}C and *Mycoplasma* (B2) showing a single peak.

Supplementary Table 4-18: Kruskal-Wallis results of abundance of *Mycoplasma* in QX-infected compared to pre-QX and uninfected SROs.

OTU	QX-infected vs pre-QX SROs		QX-infected vs uninfected SROs	
	H	**p value**	**H**	**p value**
qPCR data (numbers of copies per mg of tissue)				
	26.88	2.17E-07	4.195	0.041
16S rRNA gene sequencing data (relative abundance)				
OTU 11355	29.23	6.43E-08	13.35	0.0003
OTU 11453	17.76	2.51E-05	2.25	0.134
OTU 11532	4.778	0.03	0.5662	0.452
OTU 14055	12.09	0.0005	2.388	0.122
OTU 12128	7.298	0.007	0.7419	0.389
OTU 11599	44.12	3.09E-11	11.26	0.0008
OTU 14021	EV	EV	EV	EV
OTU 14108	17.42	2.99E-05	2.521	0.112
OTU 10424	0.4309	0.512	2.704	0.100
OTU 10420	2.107	0.147	1.37	0.242

*EV: all equal values (no variance)

Supplementary Table 4-19: Kruskal-Wallis results of abundance of *Mycoplasma* in QX-infected compared to pre-QX and uninfected SROs for each family.

Family	QX-infected vs pre-QX SROs		QX-infected vs uninfected SROs	
	H	**p value**	**H**	**p value**
OTU 11355: 16S rRNA gene sequencing data (relative abundance)				
F32	7.43	0.0064	8.727	0.0031
F43	9.18	0.003	3.975	0.046
F48	12.37	0.005	2.052	0.152
F67	4.131	0.042	5.28	0.022
qPCR data (numbers of copies per mg of tissue)				
F32	9.991	0.002	0.2591	0.611
F43	6.075	0.014	0.5526	0.457
F48	1.079	0.299	4.034	0.045
F67	17.18	3.39E-05	2.948	0.086

Supplementary Table 4-20: Kruskal-Wallis results of abundance of *Borrelia* in QX-infected compared to pre-QX and uninfected SROs.

OTU	QX-infected vs pre-QX SROs		QX-infected vs uninfected SROs	
	H	**p value**	**H**	**p value**
qPCR data (numbers of copies per mg of tissue)				
	61.2	5.16E-15	4.16	0.0414
16S rRNA gene sequencing data (relative abundance)				
OTU 1	36.68	1.39E-09	25.24	5.06E-07
OTU 10	2.917	0.088	3.12	0.077
OTU 10099	EV	EV	EV	EV
OTU 12136	0.1457	0.703	0.3779	0.539
OTU 20	0.9944	0.319	0.9105	0.34
OTU 11599	EV	EV	EV	EV

*EV: all equal values (no variance)

Supplementary Table 4-21: Kruskal-Wallis results of abundance of *Borrelia* in QX-infected compared to pre-QX and uninfected SROs for each family.

Family	QX-infected vs pre-QX SROs		QX-infected vs uninfected SROs	
	H	**p value**	**H**	**p value**
OTU 1: 16S rRNA gene sequencing data (relative abundance)				
F32	17.24	3.29E-05	9.544	0.002
F43	4.003	0.045	6.722	0.01
F48	11.9	0.001	4.238	0.04
F67	7.063	0.008	5.344	0.021
qPCR data (numbers of copies per mg of tissue)				
F32	10.71	0.001	0.3651	0.546
F43	17	3.42E-05	0.4802	0.488
F48	19.26	1.14E-05	4.321	0.038
F67	18.61	1.6E-05	3.884	0.049

Chapter 5 General discussion and conclusions

5.1 Introduction

The overall objective of this thesis was to characterise the Sydney rock oyster (SRO; *Saccostrea glomerata*) microbiota and investigate its relationship to Queensland unknown (QX) disease. In achieving this objective, this thesis provides new knowledge of the SRO microbiota and the factors that influence its assemblage. This thesis also contributes valuable information on how the SRO microbiota shifts during a QX disease event identifying key taxa that may be important for, or good indicators of, SRO health and disease. First, it was determined that the SRO microbiota is influenced by geographic location, season (comparing summer and winter) and genetics (breeding SROs for QX disease resistance) (Chapter 2). Specific OTUs were identified as being over-represented in QX resistant and susceptible oysters. Second, the SRO microbiome of QX-infected SROs was characterised and compared to those sampled prior to the QX outbreak and those that were PCR negative for the QX pathogen during the outbreak (Chapter 3). Specific OTUs associated with SRO health states were identified, an OTU assigned to *Mycoplasma* genus was associated with SROs sampled prior to the QX outbreak, while OTUs assigned to *Candidatus Hepatoplasma* and *Borrelia* genera were associated with QX-infected SROs. Additionally, the impact of QX disease progression (early infection versus late disease state) on the SRO microbiome was determined not be a factor in the SRO microbiota (Chapter 3). Third, the core microbiota was identified across four SRO families with intermediate QX disease resistance to identify taxa indicative of QX disease or, QX susceptibility and QX resistance. Two core OTUs assigned to the *Mycoplasma* and *Borrelia* genera (Chapter 4) were selected and used to design preliminary qPCR assays for investigating patterns in their absolute quantity. In this final chapter (Chapter 5), two main themes drawn from all the data in this thesis will be discussed comprising of, i) new insights into the SRO microbiota and factors affecting its assemblage and ii) a possible

role for the SRO microbiota in QX disease. Finally, possible future directions of this research will be discussed.

5.2 New insights into the SRO microbiota and the factors affecting its assemblage

Studies have identified location, season, temperature and genetics as factors influencing the microbiome composition of oysters (King *et al.*, 2012; King *et al.*, 2020; Lokmer & Wegner, 2015; Lokmer *et al.*, 2016a; Ossai *et al.*, 2017; Pierce *et al.*, 2016; Roterman *et al.*, 2015; Trabal *et al.*, 2012; Zurel *et al.*, 2011). However, the impact of location, season and genetics on the SRO microbiota have not been previously reported. Chapter 2 identified geographic location, season and genetics as having an influencing role on the SRO microbiota. Additionally, the results of chapters 3 and 4 corroborated the influence of season and genetics on the SRO microbiota assemblage. Specifically, chapter 3 showed that SROs sampled over autumn and summer had shifting microbiotas likely driven by changing environmental parameters such as temperature, nitrate and phosphate. With regard to SRO genetics, chapter 4 showed that four families with intermediate resistance to QX significantly varied in their microbiota. This is interesting because the families were similar in their phenotypic resistance to QX disease indicating that other underlying genetic factors influence the microbiota. Additionally, chapter 2 determined that the SRO microbiota did not differ between QX-resistant and QX-susceptible groups in summer however, this was not the case for the SROs in chapter 4. It should be pointed out that the SROs from chapter 4 were deployed in a different location to those from chapter 2 and that this location is prone to seasonal QX disease outbreaks. This difference can be explained by a previous study on the Pacific oyster (*Crassostrea gigas*) which demonstrated that the influence of oyster genetics on the oyster microbiota can be superseded by environmental parameters such as temperature (Wegner *et al.*, 2013). Therefore, it can be

safely stated that although the SRO microbiota is influenced by oyster genetics, it is only one of a multitude of factors with influence and and the microbiota is is shaped by a synergistic interaction of host genetics and environmental drivers.

5.3 A possible role for SRO microbiota in QX disease

Queensland Unknown (QX) disease is caused by a spore-forming protozoan parasite called *Marteilia sydneyi* (Kleeman *et al.*, 2002; Nell, 2007; Wolf, 1979) however, the presence of the pathogen does not necessarily lead to a disease outbreak (Adlard & Wesche, 2005; Department of Agriculture, Water and the Environment, 2020) indicating that other factors, beyond the presence of the pathogen, are important for infection or progression of disease. In this thesis, the microbiota was investigated as a possible factor and while the data in this thesis showed correlations between certain taxa and QX disease, it did not show causation. This thesis is the first characterising the SRO microbiota and investigating its role in QX disease and has laid a solid foundation for targeting specific taxa for investigating a putative role in QX disease or as being indicators for QX-infected/susceptible or healthy SROs. Future studies are required to conclusively demonstrate a role (if any) for the SRO microbiota in host susceptibility or resistance to QX disease. These studies are worth pursuing as studies on Pacific oysters have shown a role of the microbiota in disease (King *et al.*, 2019b; King *et al.*, 2019c; Lemire *et al.*, 2015; Pathirana *et al.*, 2019; Petton *et al.*, 2015). For example, Pacific oysters with common genetics but varying microbiota composition have different mortality outcomes when challenged with the viral pathogen OsHV-1 (Pathirana *et al.*, 2019). Additionally, a high load of the viral pathogen OsHV-1 µvar cause less disease in Pacific oysters that have had their microbiota destroyed using antibiotics (Petton *et al.*, 2015).

Importantly, the results of this thesis showed shifts in the relative abundance of OTUs assigned to *Mycoplasma*, *Candidatus Hepatoplasma* and *Borrelia* genera in relation to QX disease (Chapter 3). Specifically, *Mycoplasma* was over-represented in SROs prior to QX infection and those that were uninfected during the QX outbreak compared to QX infected SROs. Additionally, an OTU assigned to the *Candidatus Hepatoplasma* and *Borrelia* genera were over-represented in QX infected SROs (Chapter 3). These data were supported by the results in chapter 2 that showed OTUs assigned to *Mycoplasma* and *Candidatus Hepatoplasma* genera as the main drivers of microbiota dissimilarity between SROs that were genetically resistant and susceptible to QX disease respectively but was not the case for *Borrelia* genus (Chapter 2). The results in Chapter 2 showed that *Borrelia* was not over-represented in the QX- sensitive SROs. This can be explained by a previous study on Pacific oysters which indicated that the presence of *Borrelia* is influenced by location (Lasa *et al.*, 2019) which might override the influence of genetics on the relative abundance of this member in SROs. Previous studies have indicated that *Mycoplasma* is consistently dominant in the microbiota composition of healthy oysters (Green & Barnes, 2010; King *et al.*, 2012; King *et al.*, 2019b; King *et al.*, 2019c; Wegner *et al.*, 2013) and in combination with the results of this thesis, should be investigated as a possible protective bacterium. Regarding *Candidatus Hepatoplasma*, to date, there are no studies relating to its presence in oysters and so further studies are required to determine the range of this genus in other oyster species and whether it has a role in QX disease in SROs. *Borrelia* has previously been found in Pacific oysters and SROs (Green & Barnes, 2010; Lasa *et al.*, 2019), this member belongs to the Spirochaete phylum, which has been implicated as the causative agents of Pearl oyster (*Pinctada fucata martensii*) disease (Matsuyama *et al.*, 2017). In concert with the findings in this thesis, further studies are needed to determine whether *Borrelia* plays a role in QX disease in SROs.

5.4 Future directions

QX disease is a major factor in decreased SRO production (Schrobback *et al.*, 2014). Future advances in our understanding of this oyster diseases will hopefully allow us to predict and control QX disease outbreaks. As previously mentioned, the impact of QX differs across oyster estuaries with some having never experienced this disease despite the presence of the pathogen (Adlard & Wesche, 2005; Department of Agriculture, 2019). In this thesis, we demonstrated the SRO microbiota differs between two locations and two seasons (Chapter 2) however, further studies should be conducted to confirm these findings and replicate the amount of locations and seasons sampled. Additionally, oysters were sampled from two non-QX disease areas therefore, future studies should explore the SRO microbiota across multiple oyster culture areas, including QX disease and non-QX disease areas.

The QX parasite initially infects the oyster's palps and gills, then migrates through connective tissue and the haemolymph into the digestive gland where the parasite undergoes sporulation, forming mature sporonts (Kleeman *et al.*, 2002; Wolf, 1979). The results of chapter 3 and 4 showed that the adductor muscle microbiota of QX-infected SROs was significantly different when comparing to pre-QX and uninfected SROs. However, there is evidence that the oyster microbiota differs between tissues including gills, adductor muscle and the digestive gland (King *et al.*, 2012; King *et al.*, 2020; Lokmer *et al.*, 2016b) raising the question of how the digestive gland microbiota of SROs changes during an QX disease event. This might reveal additional insight.

In this thesis, a possible role for the SRO microbiota was investigated identifying specific taxa that may have a protective role, a role in facilitating QX disease or that may be good indicators of QX susceptibility or resistance. The next step is to investigate how these taxa interact with

the SRO host using culture-based methods or methods that allow visualisation of these taxa inside the tissue of the SRO host (e.g., Fluorescent *in situ* hybridisation). Culturing of these taxa could be used to identify the metabolic functionality of the microbiota however, this is challenging because they may be unculturable using current methods. Another approach to investigate the functional role of the SRO microbiota is metagenomics (Ghosh *et al.*, 2019; Handelsman, 2004; Storey *et al.*, 2020) however, there are issues associated with separating host DNA from microbiota DNA before sequencing. Methods that enrich bacteria DNA such as magnetic breads and magnetic nanoparticles (Sande *et al.*, 2020) or deep sequencing could help get around this problem but are expensive.

Predicting QX disease needs us to better understand the factors (e.g., environment, oyster genetics) that come together that tip the scales in making an SRO sensitive to QX infection. In this thesis, we have made the first step noting possible taxa that may be good indicators of QX disease resistance or susceptibility. In this thesis, preliminary qPCR assays were developed to quantify the abundance of *Mycoplasma* and *Borrelia* (Chapter 4). Overall, qPCR of *Borrelia* may be a good indicator of QX disease in SROs however, the shift patterns were not consistent across families. Additionally, the *Mycoplasma* shift patterns were not consistent with the 16S rRNA amplicon sequencing. There are many factors affecting qPCR performance for quantifying groups of taxa or specific OTUs, particularly having specific primers (Kralik & Ricchi, 2017) however, access to sufficient sequence data is necessary for efficient primer design. A limitation of our primer design was the use of a small sequence of the 16S rRNA genes which limited our capacity to design specific primers (Johnson *et al.*, 2019; Jovel *et al.*, 2016). Access to the full length of 16S rRNA gene sequences for the target OTUs and related OTUs would aid development of a qPCR assay and there is a recently developed method for doing this (Burke & Darling, 2016). An accurate qPCR assay could be useful for identifying

danger periods or even identifying SRO families that are more likely to be QX resistant, future studies should aim to improve the qPCR assays for measuring the abundance of *Mycoplasma* and *Candidatus Hepatoplasm*a in SROs.

References

Adams, A., & Thompson, K. D. (2011). Development of diagnostics for aquaculture: challenges and opportunities. *Aquac. Res., 42*(s1), 93-102. doi:https://doi.org/10.1111/j.1365-2109.2010.02663.x

Adlard, R. D., & Ernst, I. (1995). Extended range of the oyster pathogen *Marteilia sydneyi*. *Bull. Eur. Assoc. Fish. Pathol., 15*(4), 119-121.

Adlard, R. D., & Lester, R. J. G. (1995). Development of a diagnostic test for *Mikrocytos roughleyi*, the aetiological agent of Australian winter mortality of the commercial rock oyster, *Saccostrea commercialis* (Iredale & Roughley). *J. Fish Dis., 18*(6), 609-614. doi:10.1111/j.1365-2761.1995.tb00365.x

Adlard, R. D., & Wesche, S. (2005). *Aquatic Animal Health Subprogram: Development of a disease zoning policy for Marteilia sydneyi to support sustainable production, health certification and trade in the Sydney rock oyster*. Retrieved from http://www.frdc.com.au/Archived-Reports/FRDC%20Projects/2001-214-DLD.pdf

Adlard, R. D., & Nolan, M. J. (2015). Elucidating the life cycle of *Marteilia sydneyi*, the aetiological agent of QX disease in the Sydney rock oyster (*Saccostrea glomerata*). *Int. J. Parasitol., 45*(6), 419-426. doi:http://dx.doi.org/10.1016/j.ijpara.2015.02.002

Ainsworth, T. D., Krause, L., Bridge, T., Torda, G., Raina, J.-B., Zakrzewski, M., Gates, R. D., Padilla-Gamiño, J. L., Spalding, H. L., Smith, C., et al. (2015). The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *ISME J., 9*(10), 2261-2274. doi:10.1038/ismej.2015.39

Akoglu, H. (2018). User's guide to correlation coefficients. *Turk. J. Emerg. Med., 18*(3), 91-93. doi:10.1016/j.tjem.2018.08.001

Aladaileh, S., Nair, S. V., & Raftos, D. A. (2007a). Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associate molecular patterns. *Fish Shellfish Immun., 23*(6), 1196-1208. doi:https://doi.org/10.1016/j.fsi.2007.05.003

Aladaileh, S., Rodney, P., Nair, S. V., & Raftos, D. A. (2007b). Characterization of phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*). *Comp. Biochem. Physiol. B, Biochem. Mol. Biol., 148*(4), 470-480. doi:10.1016/j.cbpb.2007.07.089

Aladaileh, S., Nair, S. V., Birch, D., & Raftos, D. A. (2007c). Sydney rock oyster (*Saccostrea glomerata*) hemocytes: morphology and function. *J. Invertebr. Pathol., 96*(1), 48-63. doi:10.1016/j.jip.2007.02.011

Albanese, D., Riccadonna, S., Donati, C., & Franceschi, P. (2018). A practical tool for maximal information coefficient analysis. *Gigascience, 7*(4), 1-8. doi:10.1093/gigascience/giy032

Alsina, M., & Blanch, A. R. (1994). A set of keys for biochemical identification of environmental Vibrio species. *J. Appl. Microbiol., 76*(1), 79-85.

Anderson, T. J., Wesche, S., & Lester, R. J. G. (1994). Are outbreaks of *Marteilia sydneyi* in Sydney rock oysters, *Saccostrea commercialis*, triggered by a drop in environmental pH? *Mar. Freshw. Res., 45*(7), 1285-1287. Retrieved from https://doi.org/10.1071/MF9941285

Arias, C. R., Macian, M. C., Aznar, R., Garay, E., & Pujalte, M. J. (1999). Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. *J. Appl. Microbiol., 86*(1), 125-134. doi:10.1046/j.1365-2672.1999.00641.x

Aronson, H. S., Zellmer, A. J., & Goffredi, S. K. (2017). The specific and exclusive microbiome of the deep-sea bone-eating snail, *Rubyspira osteovora*. *FEMS microbiology ecology, 93*(3). doi:10.1093/femsec/fiw250

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Rev. Mol. Diagn., 5*(2), 209-219. doi:10.1586/14737159.5.2.209

Astudillo-Garcia, C., Bell, J. J., Webster, N. S., Glasl, B., Jompa, J., Montoya, J. M., & Taylor, M. W. (2017). Evaluating the core microbiota in complex communities: A systematic investigation. *Environ. Microbiol., 19*(4), 1450-1462. doi:10.1111/1462-2920.13647

Bakkiyaraj, D., Sivasankar, C., & Pandian, S. K. (2013). Anti-pathogenic Potential of Coral Associated Bacteria Isolated from Gulf of Mannar Against *Pseudomonas aeruginosa*. *Indian J. Microbiol., 53*(1), 111-113. doi:10.1007/s12088-012-0342-3

Bayer, T., Neave, M. J., Alsheikh-Hussain, A., Aranda, M., Yum, L. K., Mincer, T., Hughen, K., Apprill, A., & Voolstra, C. R. (2013). The microbiome of the Red sea coral

Stylophora pistillata is dominated by tissue-associated *Endozoicomonas* bacteria. *Appl. Environ. Microbiol., 79*(15), 4759. doi:10.1128/AEM.00695-13

Bayne, B. L. (2017). Metabolic Expenditure. In *Biology of Oysters* (Vol. 41, pp. 331-416).

Beaz‐Hidalgo, R., Balboa, S., Romalde, J. L., & Figueras, M. J. (2010). Diversity and pathogenecity of Vibrio species in cultured bivalve molluscs. *Environ. Microbiol. Rep., 2*(1), 34-43.

Bezemer, B., Butt, D., Nell, J., Adlard, R., & Raftos, D. (2006). Breeding for QX disease resistance negatively selects one form of the defensive enzyme, phenoloxidase, in Sydney rock oysters. *Fish Shellfish Immun., 20*(4), 627-636. doi:10.1016/j.fsi.2005.08.007

Boettcher, K. J., Barber, B. J., & Singer, J. T. (2000). Additional evidence that juvenile oyster disease is caused by a member of the *Roseobacter* group and colonization of nonaffected animals by *Stappia stellulata-Like* strains. *Appl. Environ. Microbiol., 66*(9), 3924-3930.

Brown, M. R., Barrett, S. M., Volkman, J. K., Nearhos, S. P., Nell, J. A., & Allan, G. L. (1996). Biochemical composition of new yeasts and bacteria evaluated as food for bivalve aquaculture. *Aquaculture, 143*(3), 341-360. doi:http://dx.doi.org/10.1016/0044-8486(96)01286-0

Burke, C. M., & Darling, A. E. (2016). A method for high precision sequencing of near full-length 16S rRNA genes on an Illumina MiSeq. *PeerJ, 4*, e2492. doi:10.7717/peerj.2492

Butt, D., Shaddick, K., & Raftos, D. (2006). The effect of low salinity on phenoloxidase activity in the Sydney rock oyster, *Saccostrea glomerata*. *Aquaculture, 251*(2), 159-166.

Butt, D., & Raftos, D. (2007). Immunosuppression in Sydney rock oysters (*Saccostrea glomerata*) and QX disease in the Hawkesbury River, Sydney. *Mar. Freshwater Res., 58*(2), 213-221. doi:https://doi.org/10.1071/MF06080

Butt, D., & Raftos, D. (2008). Phenoloxidase-associated cellular defence in the Sydney rock oyster, *Saccostrea glomerata*, provides resistance against QX disease infections. *Dev. Comp. Immunol., 32*(3), 299-306. doi:10.1016/j.dci.2007.06.006

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., et al. (2010). QIIME allows

analysis of high-throughput community sequencing data. *Nat. Methods, 7*(5), 335-336. doi:10.1038/nmeth.f.303

Carnegie, R. B., & Cochennec-Laureau, N. (2004). Microcell parasites of oysters: Recent insights and future trends. *Aquat. Living Resour., 17*(4), 519-528. Retrieved from http://dx.doi.org/10.1051/alr:2004055

Cerenius, L., & Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunol. Rev., 198*(1), 116-126. doi:10.1111/j.0105-2896.2004.00116.x

Cerf-Bensussan, N., & Gaboriau-Routhiau, V. (2010). The immune system and the gut microbiota: friends or foes? *Nat. Rev. Immunol., 10*(10), 735-744.

Chauhan, A., Wafula, D., Lewis, D. E., & Pathak, A. (2014). Metagenomic assessment of the Eastern oyster-associated microbiota. *Genome announc., 2*(5), e01083-01014.

Cochennec, N., Reece, K., Berthe, F., & Hine, M. (2003). *Mikrocytos roughleyi* taxonomic affiliation leads to the genus Bonamia (Haplosporidia). *Dis. Aquat. Org., 54*(3), 209-217.

Colwell, R. R., & Liston, J. (1960). Microbiology of shellfish. Bacteriological study of the natural flora of Pacific oysters (*Crassostrea gigas*). *Appl. Microbiol., 8*(2), 104-109. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1057579/

Crosby, M. P., Newell, R. I., & Langdon, C. J. (1990). Bacterial mediation in the utilization of carbon and nitrogen from detrital complexes by *Crassostrea virginica*. *Limnol. Oceanogr., 35*(3), 625-639.

da Silva, P. M., & Villalba, A. (2004). Comparison of light microscopic techniques for the diagnosis of the infection of the European flat oyster *Ostrea edulis* by the protozoan *Bonamia ostreae*. *J. Invertebr. Pathol., 85*(2), 97-104. doi:http://dx.doi.org/10.1016/j.jip.2003.12.010

De Leeuw, B. H. C. G. M., Maraha, B., Hollemans, L., Sprong, H., Brandenburg, A. H., Westenend, P. J., & Kusters, J. G. (2014). Evaluation of *Borrelia* real time PCR DNA targeting OspA, FlaB and 5S–23S IGS and *Borrelia* 16S rRNA RT-qPCR. *J. Microbiol. Methods, 107*, 41-46. doi:https://doi.org/10.1016/j.mimet.2014.09.001

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., et al. (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat. Commun., 9*(1), 4215. doi:10.1038/s41467-018-06659-3

Defer, D., Desriac, F., Henry, J., Bourgougnon, N., Baudy-Floc'h, M., Brillet, B., Le Chevalier, P., & Fleury, Y. (2013). Antimicrobial peptides in oyster hemolymph: The bacterial

connection. *Fish Shellfish Immun., 34*(6), 1439-1447. doi:https://doi.org/10.1016/j.fsi.2013.03.357

del Refugio Castañeda Chávez, M., Sedas, V. P., Orrantia Borunda, E., & Reynoso, F. L. (2005). Influence of water temperature and salinity on seasonal occurrences of *Vibrio cholerae* and enteric bacteria in oyster-producing areas of Veracruz, México. *Mar. Pollut. Bull., 50*(12), 1641-1648. doi:10.1016/j.marpolbul.2005.06.036

Department of Agriculture, Water and the Environment. (2020). *Aquatic animal diseases significant to Australia: Identification field guide, 5th edition*, Australian Government Department of Agriculture, Water and the Environment, Canberra. CC BY 4.0.

Department of Primary Industries. (2016). *NSW oyster industry sustainable aquaculture strategy*. NSW: Department of Primary Industries Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/638250/NSW-oyster-industry-sustainable-aquaculture-strategy-2016.pdf

Desriac, F., Le Chevalier, P., Brillet, B., Leguerinel, I., Thuillier, B., Paillard, C., & Fleury, Y. (2014). Exploring the hologenome concept in marine bivalvia: haemolymph microbiota as a pertinent source of probiotics for aquaculture. *FEMS microbiol. Lett., 350*(1), 107-116.

Diggles, B. K. (2013). Historical epidemiology indicates water quality decline drives loss of oyster (*Saccostrea glomerata*) reefs in Moreton Bay, Australia. *New Zeal. J. Mar. Fresh., 47*(4), 561-581. doi:10.1080/00288330.2013.781511

Dinamani, P. (1974). Reproductive cycle and gonadial changes in the New Zealand rock oyster *Crassostrea glomerata*. *New Zeal. J. Mar. Fresh., 8*(1), 39-65. doi:10.1080/00288330.1974.9515490

Dove, M., Kube, P., Wilkie, E., Cumbo, V., Raftos, D., O'Connor, W., & New Soth Wales Department of Primery Industries (Fisheries). (2020). *Accelerated Sydney rock oyster (SRO) breeding research. Future oysters CRC-P Project 2016-802*. Retrieved from Taylors Beach:

Dove, M. C., & O'connor, W. A. (2007). Salinity and temperature tolerance of Sydney rock oysters *Saccostrea glomerata* during early ontogeny. *J. Shellfish Res., 26*(4), 939-947.

Dove, M. C., & Sammut, J. (2007a). Histological and feeding response of Sydney rock oysters, *Saccostrea glomerata*, to acid sulfate soil outflows. *J. Shellfish Res., 26*(2), 509-518.

Dove, M. C., & Sammut, J. (2007b). Impacts of estuarien acidification on survival and growth of Sydney Rock Oysters *Saccostrea Glomerata* (Gould 1850). *J. Shellfish Res., 26*(2), 519-527. doi:10.2983/0730-8000(2007)26[519:IOEAOS]2.0.CO;2

Dove, M. C., & O'Connor, W. A. (2012). Reproductive cycle of Sydney rock oysters, *Saccostrea glomerata* (Gould 1850) selectively bred for faster growth. *Aquaculture, 324–325*, 218-225. doi:http://dx.doi.org/10.1016/j.aquaculture.2011.10.022

Dove, M. C., Nell, J. A., & O'Connor, W. A. (2013a). Evaluation of the progeny of the fourth‐generation Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) breeding lines for resistance to QX disease (*Marteilia sydneyi*) and winter mortality (*Bonamia roughleyi*). *Aquac. Res., 44*(11), 1791-1800.

Dove, M. C., Nell, J. A., Mcorrie, S., & O'connor, W. A. (2013b). Assessment of Qx and winter mortality disease resistance of mass selected Sydney rock oysters, *Saccostrea glomerata* (Gould, 1850), in the Hawkesbury River and Merimbula Lake, NSW Australia. *J. Shellfish Res., 32*(3), 681-687.

Dubé, C. E., Ky, C.-L., & Planes, S. (2019). Microbiome of the Black-lipped pearl oyster *Pinctada margaritifera*, a multi-tissue description with functional profiling. *Front. Microbiol., 10*(1548). doi:10.3389/fmicb.2019.01548

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

El-Shanshoury, A. R., Mona, M. H., Shoukr, F. A., & El-Bossery, A. M. (1994). The enumeration and characterization of bacteria and fungi associated with marine wood-boring isopods, and the ability of these microorganisms to digest cellulose and wood. *Mar. Biol., 119*(3), 321-326. doi:10.1007/BF00347528

Elston, R., & Leibovitz, L. (1980). Pathogenesis of experimental vibriosis in larval American oysters, *Crassostrea virginica*. *Can. J. Fish. Aquat. Sci., 37*(6), 964-978.

Erasmus, J. H., Cook, P. A., & Coyne, V. E. (1997). The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture, 155*(1), 377-386.

Estes, R. M., Friedman, C. S., Elston, R. A., & Herwig, R. P. (2004). Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. *Dis. Aquat. Organ., 58*(2-3), 223-230.

Evseev, G. A., Yakovlev, Y. M., & Li, X. (1996). The Anatomy of the Pacific Oyster, *Crassostrea gigas* (Thurnberg) (Bivalvia: Ostreidae). *Publ. Seto Mar. Biol. Lab., 37*(3-6), 239-255.

FAO. (2005). Cultured aquatic species information programme. *Saccostrea commercialis*. Cultured aquatic species information programme. Retrieved from http://www.fao.org/fishery/culturedspecies/Saccostrea_commercialis/en

Farley, C. A., Wolf, P. H., & Elstons, R. A. (1988). A long-term study of "microcell" disease in oysters with a description of a new genus, *Mikrocytos* (g. n.), and two new species, *Mikrocytos mackini* (sp. n.) and *Mikrocytos roughleyi* (sp. n.). *Fish. Bull., 86*(3), 581-593.

Froelich, B. A., Phippen, B., Fowler, P., Noble, R. T., & Oliver, J. D. (2016). Differences in Abundances of Total *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* in Clams and Oysters in North Carolina. *Appl. Environ. Microbiol., 83*(2), e02265-02216. doi:10.1128/AEM.02265-16

Gagnaire, B., Duchemin, M., Auffret, M., Thomas-Guyon, H., & Renault, T. (2008). Comparison of hemocyte parameters in the pericardial cavity and the adductor muscle sinus in the Pacific oyster, *Crassostrea gigas* using two types of flow cytometers. *Aquat. Living Resour., 21*(1), 39-43. Retrieved from https://doi.org/10.1051/alr:2008009

Galtsoff, P. S. (1964). *The American oyster, Crassostrea virginica Gmelin* (Vol. 64). Washington [D.C.]: Fishery Bulletin of the Fish and Wildlife Service.

Garland, C. D., Nash, G. V., Summer, C. E., & McMeekin, T. A. (1983). Bacterial pathogens of oyster larvae (*Crassostrea gigas*) in a Tasmanian Hatchery. *Mar. Freshwater Res., 34*(3), 483-487. Retrieved from https://doi.org/10.1071/MF9830483

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., & Nicolas, J.-L. (2007). Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. *Microb. Ecol., 53*(2), 187-196. doi:10.1007/s00248-006-9061-9

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture, 180*(1–2), 147-165. doi:http://dx.doi.org/10.1016/S0044-8486(99)00187-8

Gay, M., Berthe, F. C., & Le Roux, F. (2004). Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis. Aquat. Organ., 59*(1), 49.

Ghosh, A., Mehta, A., & Khan, A. M. (2019). Metagenomic Analysis and its Applications. In S. Ranganathan, M. Gribskov, K. Nakai, & C. Schönbach (Eds.), *Encyclopedia of Bioinformatics and Computational Biology* (pp. 184-193). Oxford: Academic Press.

Gillies, C. L., McLeod, I. M., Alleway, H. K., Cook, P., Crawford, C., Creighton, C., Diggles, B., Ford, J., Hamer, P., Heller-Wagner, G., et al. (2018). Australian shellfish ecosystems: Past distribution, current status and future direction. *PloS One, 13*(2), e0190914. doi:10.1371/journal.pone.0190914

Gosling, E. (2015). Morphology of bivalves. In E. Gosling (Ed.), *Marine Bivalve Molluscs* (pp. 12-43). Retrieved from https://onlinelibrary.wiley.com/doi/10.1002/9781119045212.ch2

Green, T. J., Dixon, T. J., Devic, E., Adlard, R. D., & Barnes, A. C. (2009). Differential expression of genes encoding anti-oxidant enzymes in Sydney rock oysters, *Saccostrea glomerata* (Gould) selected for disease resistance. *Fish Shellfish Immun., 26*(5), 799-810. doi:https://doi.org/10.1016/j.fsi.2009.03.003

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *J. Appl. Microbiol., 109*(2), 613-622. doi:10.1111/j.1365-2672.2010.04687.x

Green, T. J., Raftos, D., O'Connor, W., Adlard, R. D., & Barnes, A. C. (2011). Disease prevention strategies for QX disease (*Marteilia sydneyi*) of Sydney rock oysters (*Saccostrea glomerata*). *J. Shellfish Res., 30*(1), 47-53.

Hammer, Øyvind, Harper, David, A. T., & Paul, D. R. (2001). Past: Paleontological statistics software package for education and data analysis. *Palaeontol. Electronica, 4*(1), 9. Retrieved from https://palaeo-electronica.org/2001_1/past/past.pdf

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. R., 68*(4), 669-685. doi:10.1128/MMBR.68.4.669-685.2004

Hasegawa, H., Lind, E. J., Boin, M. A., & Häse, C. C. (2008). The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for pacific oyster (Crassostrea gigas) larvae. *Appl. Environ. Microbiol., 74*(13), 4101-4110.

Hedrick, R. (1998). Relationships of the host, pathogen, and environment: implications for diseases of cultured and wild fish populations. *J. Aquat. Anim. Health, 10*(2), 107-111.

Helm, M. M., Bourne, N., & Lovatelli, A. (2004). *Hatchery Culture of Bivalves: A Practical Manual*(pp. 177). Retrieved from http://www.fao.org/3/a-y5720e.pdf

Hernandez-Agreda, A., Leggat, W., Bongaerts, P., & Ainsworth, T. D. (2016). The Microbial Signature Provides Insight into the Mechanistic Basis of Coral Success across Reef Habitats. *mBio, 7*(4), e00560-00516. doi:10.1128/mBio.00560-16

Hernandez-Agreda, A., Leggat, W., Bongaerts, P., Herrera, C., & Ainsworth, T. D. (2018). Rethinking the coral microbiome: Simplicity exists within a diverse microbial biosphere. *mBio, 9*(5), e00812-00818. doi:10.1128/mBio.00812-18

Huse, S. M., Ye, Y., Zhou, Y., & Fodor, A. A. (2012). A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters. *PLoS One, 7*(6), e34242. doi:10.1371/journal.pone.0034242

Husmann, G., Gerdts, G., & Wichels, A. (2010). Spirochetes in crystalline styles of marine bivalves: group-specific PCR detection and 16S rRNA sequence analysis. *J. Shellfish Res., 29*(4).

Hussain, M. A., Saputra, T., Szabo, E. A., & Nelan, B. (2017). An Overview of Seafood Supply, Food Safety and Regulation in New South Wales, Australia. *Foods, 6*(7), 52. doi:10.3390/foods6070052

Jefferson, R. (2019). *Aquaculture production report 2017- 2018*. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/851342/Aquaculture-Production-Report-2017-2018.pdf

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., et al. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat. Commun., 10*(1), 5029. doi:10.1038/s41467-019-13036-1

Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A. L., Madsen, K. L., et al. (2016). Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. *Front. Microbiol., 7*, 459-459. doi:10.3389/fmicb.2016.00459

Kahlke, T. (2017). Panbiom. Retrieved from https://github.com/timkahlke/panbiom

Kamada, N., Chen, G. Y., Inohara, N., & Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nat. Immunol., 14*(7), 685-690. doi:10.1038/ni.2608

Kennedy, V. S., Newell, R. I. E., Eble, A. F., & College, M. S. G. (1996). *The Eastern Oyster: Crassostrea Virginica*: Maryland Sea Grant College.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the Eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One, 7*(12), e51475. doi:10.1371/journal.pone.0051475

King, W. L., Jenkins, C., Seymour, J. R., & Labbate, M. (2019a). Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment. *Mar. Environ. Res., 143*, 124-140. doi:10.1016/j.marenvres.2018.11.007

King, W. L., Jenkins, C., Go, J., Siboni, N., Seymour, J. R., & Labbate, M. (2019b). Characterisation of the Pacific oyster microbiome during a summer mortality event. *Microbiol. Ecol., 77*(2), 502-512. doi:10.1007/s00248-018-1226-9

King, W. L., Siboni, N., Williams, N. L. R., Kahlke, T., Nguyen, K. V., Jenkins, C., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2019c). Variability in the composition of Pacific oyster microbiomes across oyster families exhibiting different levels of

susceptibility to OsHV-1 µvar disease. *Front. Microbiol., 10*, 473-473. doi:10.3389/fmicb.2019.00473

King, W. L., Siboni, N., Kahlke, T., Green, T. J., Labbate, M., & Seymour, J. R. (2019d). A New High Throughput Sequencing Assay for Characterizing the Diversity of Natural *Vibrio* Communities and Its Application to a Pacific Oyster Mortality Event. *Front. Microbiol., 10*, 2907.

King, W. L., Siboni, N., Kahlke, T., Dove, M., O'Connor, W., Mahbub, K. R., Jenkins, C., Seymour, J. R., & Labbate, M. (2020). Regional and oyster microenvironmental scale heterogeneity in the Pacific oyster bacterial community. *FEMS Microbiol. Ecol.* doi:10.1093/femsec/fiaa054

Klaus, J. S., Frias-Lopez, J., Bonheyo, G. T., Heikoop, J. M., & Fouke, B. W. (2005). Bacterial communities inhabiting the healthy tissues of two Caribbean reef corals: interspecific and spatial variation. *Coral Reefs, 24*(1), 129-137. doi:10.1007/s00338-004-0447-1

Kleeman, S. N., & Adlard, R. D. (2000). Molecular detection of *Marteilia sydneyi,* pathogen of Sydney rock oysters. *Dis. Aquat. Organ., 40*(2), 137-146.

Kleeman, S. N., Adlard, R. D., & Lester, R. J. G. (2002). Detection of the initial infective stages of the protozoan parasite *Marteilia sydneyi* in *Saccostrea glomerata* and their development through to sporogenesis. *Int. J. Parasitol., 32*(6), 767-784. doi:http://dx.doi.org/10.1016/S0020-7519(02)00025-5

Kow, F., Yu, L., FitzGerald, D., & Grewal, D. (2008). Understanding the factors related to the consumers' choices of oysters in Australia: an empirical study. *J. Foodservice, 19*(4), 245-253. doi:10.1111/j.1748-0159.2008.00112.x

Kralik, P., & Ricchi, M. (2017). A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front. Microbiol., 8*(108). doi:10.3389/fmicb.2017.00108

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., et al. (2006). The real-time polymerase chain reaction. *Mol. Aspects Med., 27*(2), 95-125. doi:https://doi.org/10.1016/j.mam.2005.12.007

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol., 35*(6), 1547-1549. doi:10.1093/molbev/msy096

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M., & Poulet, S. (2001). A *Vibrio splendidus* strain is associated with summer mortality of

juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis. Aquat. Organ., 46*(2), 139-145.

Lane, D. J. (1991). 16S/23S rRNA Sequencing. In Stackebrandt. E & Goodfellow. M (Eds.), *Nucleic Acid Techniques in Bacterial Systematics* (pp. 115-147). West Sussex: John Wiley & Sons Ltd.

Larsen, A. M., Scott Rikard, F., Walton, W. C., & Arias, C. R. (2013). Effective reduction of *Vibrio vulnificus* in the Eastern oyster (*Crassostrea virginica*) using high salinity depuration. *Food Microbiol., 34*(1), 118-122. doi:10.1016/j.fm.2012.11.009

Lasa, A., di Cesare, A., Tassistro, G., Borello, A., Gualdi, S., Furones, D., Carrasco, N., Cheslett, D., Brechon, A., Paillard, C., et al. (2019). Dynamics of the Pacific oyster pathobiota during mortality episodes in Europe assessed by 16S rRNA gene profiling and a new target enrichment next-generation sequencing strategy. *Environ. Microbiol., 21*(12), 4548-4562. doi:10.1111/1462-2920.14750

Lawson, C. A., Raina, J. B., Kahlke, T., Seymour, J. R., & Suggett, D. J. (2018). Defining the core microbiome of the symbiotic dinoflagellate, Symbiodinium. *Environ. Microbiol. Rep., 10*(1), 7-11. doi:10.1111/1758-2229.12599

Lemire, A., Goudenege, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., & Le Roux, F. (2015). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *ISME J., 9*(7), 1523-1531. doi:10.1038/ismej.2014.233

Lester, R. J. G. (1986). Field and laboratory observations on the oyster parasite *Marteilia sydneyi*. In J. F. A. Sprent, M. Cremin, C. Dobson, & D. E. Moorhouse (Eds.), *Parasite Lives* (pp. 33-40). Bribane: University of Queensland Press.

Levican, A., Collado, L., Yustes, C., Aguilar, C., & Figueras, M. J. (2014). Higher Water Temperature and Incubation under Aerobic and Microaerobic Conditions Increase the Recovery and Diversity of *Arcobacter* spp. from Shellfish. *Appl. Environ. Microbiol., 80*(1), 385. doi:10.1128/AEM.03014-13

Liu, Y., Kow, F., Grewal, D., & FitzGerald, D. (2006). Consumer purchase behaviour for oysters: an empirical study in some state capital cities of Australia. *Int. J. Consum. Stud., 30*(1), 85-94. doi:10.1111/j.1470-6431.2005.00456.x

Livingstone, S. (2013). *Aquaculture Prodcution Report 2011 - 2012*. Retrieved from http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0007/638071/Aquaculture-Production-Report-2011-2012.pdf

Lokmer, A., & Wegner, M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J., 9*(3), 670-682. doi:10.1038/ismej.2014.160

Lokmer, A., Goedknegt, M. A., Thieltges, D. W., Fiorentino, D., Kuenzel, S., Baines, J. F., & Wegner, K. M. (2016a). Spatial and temporal dynamics of Pacific oyster hemolymph microbiota across multiple scales. *Front. Microbiol., 7*, 1367. doi:10.3389/fmicb.2016.01367

Lokmer, A., Kuenzel, S., Baines, J. F., & Wegner, K. M. (2016b). The role of tissue-specific microbiota in initial establishment success of Pacific oysters. *Environ. Microbiol., 18*(3), 970-987. doi:10.1111/1462-2920.13163

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963. doi:10.1093/bioinformatics/btr507

Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome, 3*(1), 31. doi:10.1186/s40168-015-0094-5

Margos, G., Fingerle, V., Cutler, S., Gofton, A., Stevenson, B., & Estrada-Peña, A. (2020). Controversies in bacterial taxonomy: The example of the genus Borrelia. *Ticks and Tick-borne Dis., 11*(2), 101335. doi:https://doi.org/10.1016/j.ttbdis.2019.101335

Matsuyama, T., Yasuike, M., Fujiwara, A., Nakamura, Y., Takano, T., Takeuchi, T., Satoh, N., Adachi, Y., Tsuchihashi, Y., Aoki, H., et al. (2017). A *Spirochaete* is suggested as the causative agent of Akoya oyster disease by metagenomic analysis. *PloS One, 12*(8), e0182280. doi:10.1371/journal.pone.0182280

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A., & Zenger, K. R. (2019). Methods for normalizing microbiome data: An ecological perspective. *Methods Ecol. and Evol., 10*(3), 389-400. doi:10.1111/2041-210X.13115

Meziti, A., Mente, E., & Kormas, K. A. (2012). Gut bacteria associated with different diets in reared *Nephrops norvegicus*. *Syst. Appl. Microbiol., 35*(7), 473-482. doi:10.1016/j.syapm.2012.07.004

Morrison, T. B., Ma, Y., Weis, J. H., & Weis, J. J. (1999). Rapid and sensitive quantification of *Borrelia burgdorferi*-infected mouse tissues by continuous fluorescent monitoring of PCR. *J. Clin. Microbiol., 37*(4), 987-992. Retrieved from https://pubmed.ncbi.nlm.nih.gov/10074514

Mortzfeld, B. M., Urbanski, S., Reitzel, A. M., Künzel, S., Technau, U., & Fraune, S. (2016). Response of bacterial colonization in *Nematostella vectensis* to development,

environment and biogeography. *Environ. Microbiol., 18*(6), 1764-1781. doi:10.1111/1462-2920.12926

Motes, M. L., DePaola, A., Cook, D. W., Veazey, J. E., Hunsucker, J. C., Garthright, W. E., Blodgett, R. J., & Chirtel, S. J. (1998). Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). *Appl. Environ. Microbiol., 64*(4), 1459-1465. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC106170/

Mueller, U. G., & Sachs, J. L. (2015). Engineering Microbiomes to Improve Plant and Animal Health. *Trends Microbiol., 23*(10), 606-617. doi:http://dx.doi.org/10.1016/j.tim.2015.07.009

Nadkarni, M. A., Martin, F. E., Jacques, N. A., & Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology, 148*(1), 257-266. doi:https://doi.org/10.1099/00221287-148-1-257

Nakagawa, S., Saito, H., Tame, A., Hirai, M., Yamaguchi, H., Sunata, T., Aida, M., Muto, H., Sawayama, S., & Takaki, Y. (2017). Microbiota in the coelomic fluid of two common coastal starfish species and characterization of an abundant *Helicobacte*r-related taxon. *Sci. Rep., 7*, 8764. doi:10.1038/s41598-017-09355-2

Neave, M. J., Apprill, A., Ferrier-Pagès, C., & Voolstra, C. R. (2016). Diversity and function of prevalent symbiotic marine bacteria in the genus *Endozoicomonas*. *Appl. Microbiol. Biotechnol., 100*(19), 8315-8324. doi:10.1007/s00253-016-7777-0

Neave, M. J., Michell, C. T., Apprill, A., & Voolstra, C. R. (2017). *Endozoicomonas* genomes reveal functional adaptation and plasticity in bacterial strains symbiotically associated with diverse marine hosts. *Sci. Rep., 7*, 40579. doi:10.1038/srep40579

Nell, J. (2007). *Diseases of Sydney rock oysters*. NSW, Australia: NSW Department of Primary Industries, Retrieved from https://marine-aquaculture.extension.org/wp-content/uploads/2019/05/Diseases-of-Sydney-Rock-Oysters.pdf

Nell, J. A., & Holliday, J. E. (1988). Effects of salinity on the growth and survival of Sydney rock oyster (*Saccostrea commercialis*) and Pacific oyster (*Crassostrea gigas*) larvae and spat. *Aquaculture, 68*(1), 39-44. doi:http://dx.doi.org/10.1016/0044-8486(88)90289-X

Nell, J. A. (1993). Farming the Sydney rock oyster (*Saccostrea commercialis*) in Australia. *Rev. Fish. Sci., 1*(2), 97-120. doi:10.1080/10641269309388537

Nell, J. A., Smith, I. R., & McPhee, C. C. (2000). The Sydney rock oyster *Saccostrea glomerata* (Gould 1850) breeding programme: progress and goals. *Aquaculture Res., 31*(1), 45-49.

Nell, J. A. (2001). The History of Oyster Farming in Australia. *Mar. Fish. Rev., 63*(3), 14-25.

Nell, J. A., & Perkins, B. (2006). Evaluation of the progeny of third‐generation Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) breeding lines for resistance to QX disease *Marteilia sydneyi* and winter mortality *Bonamia roughleyi*. *Aquaculture Res., 37*(7), 693-700.

Newton, K., Peters, R., & Raftos, D. (2004). Phenoloxidase and QX disease resistance in Sydney rock oysters (*Saccostrea glomerata*). *Dev. Comp. Immunol., 28*(6), 565-569. doi:10.1016/j.dci.2003.10.004

Nguyen, V. K., King, W. L., Siboni, N., Mahbub, K. R., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). The Sydney rock oyster microbiota is influenced by location, season and genetics. *Aquaculture*, 735472. doi:https://doi.org/10.1016/j.aquaculture.2020.735472

Nishijima, M., Adachi, K., Katsuta, A., Shizuri, Y., & Yamasato, K. (2013). *Endozoicomonas numazuensis* sp. nov., a gammaproteobacterium isolated from marine sponges, and emended description of the genus *Endozoicomonas Kurahashi and Yokota 2007*. *Int. J. Syst. Evol. Microbiol., 63*(Pt 2), 709-714. doi:10.1099/ijs.0.042077-0

O'Connor, W., & Dove, M. (2009). The changing face of oyster culture in New South Wales, Australia. *J. Shellfish Res., 28*, 803-811.

O'Connor, W., Dove, M. C., Thompson, E. L., Parker, L. M., Ross, P. M., & Raftos, D. A. (2014). Breeding Sydney rock oysters and its effects on resilience. In S. Hermesch (Ed.), *Breeding Focus 2014 – Improving Resilience* (pp. 73 - 86): Animal Genetics and Breeding Unit, University of New England.

O'Connor, W. A., & Newman, L. J. (2001). Halotolerance of the oyster predator, *Imogine mcgrathi,* a stylochid flatworm from Port Stephens, New South Wales, Australia. *Hydrobiologia, 459*(1), 157-163. doi:10.1023/A:1012525015850

O'Rourke, M., Traweger, A., Lusa, L., Stupica, D., Maraspin, V., Barrett, P. N., Strle, F., & Livey, I. (2013). Quantitative detection of *Borrelia burgdorferi* sensu lato in erythema migrans skin lesions using internally controlled duplex real time PCR. *PloS One, 8*(5), e63968-e63968. doi:10.1371/journal.pone.0063968

O'Connor, W., Dove, M., Finn, B., & O'Connor, S. (2008). *Manual for hatchery production of Sydney rock oysters (Saccostrea glomerata)*. Retrieved from https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0014/261041/Output-1084_Hatchery-Manual_FORMATTED.pdf

Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., Le Chevalier, P., & Fleury, Y. (2019). Protective efficacy of a *Pseudoalteromonas* strain in European abalone, *Haliotis tuberculata*, infected with *Vibrio harveyi* ORM4. *Probiotics Antimicro., 11*(1), 239-247. doi:10.1007/s12602-018-9389-8

Ogburn, D. M., White, I., & McPhee, D. P. (2007). The Disappearance of Oyster Reefs from Eastern Australian Estuaries—Impact of Colonial Settlement or Mudworm Invasion? *Coast. Manage., 35*(2-3), 271-287. doi:10.1080/08920750601169618

Olafsen, J. A., Mikkelsen, H. V., Giæver, H. M., & Høvik Hansen, G. (1993). Indigenous Bacteria in Hemolymph and Tissues of Marine Bivalves at Low Temperatures. *Appl. Environ. Microbiol., 59*(6), 1848. Retrieved from http://aem.asm.org/content/59/6/1848.abstract

Ossai, S., Ramachandran, P., Ottesen, A., Reed, E., DePaola, A., & Parveen, S. (2017). Microbiomes of American oysters (*Crassostrea virginica*) harvested from two sites in the Chesapeake bay. *Genome Announc., 5*(30), e00729-00717. doi:10.1128/genomeA.00729-17

Paillard, C., Le Roux, F., & Borrego, J. J. (2004). Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquat. Living Resour., 17*(04), 477-498.

Panetta, J. L., Šíma, R., Calvani, N. E. D., Hajdušek, O., Chandra, S., Panuccio, J., & Šlapeta, J. (2017). Reptile-associated Borrelia species in the goanna tick (*Bothriocroton undatum*) from Sydney, Australia. *Parasit. Vectors, 10*(1), 616. doi:10.1186/s13071-017-2579-5

Parker, L. M., Ross, P. M., & O'Connor, W. A. (2009). The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1850). *Glob. Change Biol., 15*(9), 2123-2136.

Parker, L. M., Ross, P. M., & O'Connor, W. A. (2011). Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. *Mar. Biol., 158*(3), 689-697. doi:10.1007/s00227-010-1592-4

Parks, D. H., Tyson, G. W., Hugenholtz, P., & Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics, 30*(21), 3123-3124. doi:10.1093/bioinformatics/btu494

Paterson, K. J., Schreider, M. J., & Zimmerman, K. D. (2003). Anthropogenic effects on seston quality and quantity and the growth and survival of Sydney rock oyster (*Saccostrea glomerata*) in two estuaries in NSW, Australia. *Aquaculture, 221*(1), 407-426. doi:https://doi.org/10.1016/S0044-8486(03)00027-9

Pathirana, E., Fuhrmann, M., Whittington, R., & Hick, P. (2019). Influence of environment on the pathogenesis of Ostreid herpesvirus-1 (OsHV-1) infections in Pacific oysters (*Crassostrea gigas*) through differential microbiome responses. *Heliyon, 5*(7), e02101. doi:https://doi.org/10.1016/j.heliyon.2019.e02101

Perkins, F. O., & Wolf, P. H. (1976). Fine structure of *Marteilia sydneyi* sp. n.: Haplosporidan pathogen of Australian oysters. *J. Parasitol., 62*(4), 528-538. doi:10.2307/3279407

Peters, R., & Raftos, D. A. (2003). The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). *Aquaculture, 223*(1), 29-39. doi:10.1016/S0044-8486(03)00169-8

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., & Le Roux, F. (2015). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front. Microbiol., 6*, 686. doi:10.3389/fmicb.2015.00686

Pierce, M. L., Ward, J. E., Holohan, B. A., Zhao, X., & Hicks, R. E. (2016). The influence of site and season on the gut and pallial fluid microbial communities of the eastern oyster, *Crassostrea virginica* (Bivalvia, Ostreidae): community-level physiological profiling and genetic structure. *Hydrobiologia, 765*(1), 97-113.

Pierce, M. L., & Ward, J. E. (2019). Gut Microbiomes of the Eastern oyster (*Crassostrea virginica*) and the Blue mussel (*Mytilus edulis*): Temporal variation and the influence of marine aggregate-associated microbial communities. *mSphere, 4*(6), e00730-00719. doi:10.1128/mSphere.00730-19

Prado, S., Montes, J., Romalde, J. L., & Barja, J. L. (2009). Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *Int. Microbiol., 12*(2), 107-114.

Prieur, D., Mevel, G., Nicolas, J.-L., Plusquellec, A., & Vigneulle, M. (1990). Interactions between bivalve molluscs and bacteria in the marine environment. *Oceanogr. Mar. Biol. Annu. Rev., 28*, 277-352.

Pujalte, M., Ortigosa, M., Macián, M., & Garay, E. (1999). Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *Int. Microbiol., 2*(4), 259 -266.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res., 41*(Database issue), D590-D596. doi:10.1093/nar/gks1219

Raftos, D. A., Kuchel, R., Aladaileh, S., & Butt, D. (2014). Infectious microbial diseases and host defense responses in Sydney rock oysters. *Front. Microbiol., 5*, 135. doi:10.3389/fmicb.2014.00135

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America, 101*(13), 4596-4601. doi:10.1073/pnas.0400706101

Read, G. B. (2010). Comparison and history of *Polydora websteri* and *P. haswelli* (Polychaeta: Spionidae) as mud-blister worms in New Zealand shellfish. *New Zeal. J. Mar. Fresh., 44*(2), 83-100. doi:10.1080/00288330.2010.482969

Reid, G., Howard, J., & Gan, B. S. (2001). Can bacterial interference prevent infection? *Trends Microbiol., 9*(9), 424-428. doi:10.1016/S0966-842X(01)02132-1

Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosyn. Res., 89*(1), 27-41. doi:10.1007/s11120-006-9065-9

Roder, C., Arif, C., Daniels, C., Weil, E., & Voolstra, C. R. (2014). Bacterial profiling of White plague disease across corals and oceans indicates a conserved and distinct disease microbiome. *Mol. Ecol., 23*(4), 965-974. doi:10.1111/mec.12638

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ, 4*, e2584. doi:10.7717/peerj.2584

Romero, J., & Espejo, R. (2001). The prevalence of noncultivable bacteria in oysters (*Tiostrea chilensis*, Philippi, 1845). *J. Shellfish Res., 20*(3), 1235 - 1240.

Roper, T., Creese, B., Scanes, P., Stephens, K., Williams, R., Dela-Cruz, J., Coade, G., Coates, B., & Fraser, M. (2011). *Assessing the condition of estuaries and coastal lake ecosystems in NSW*. Retrieved from https://www.environment.nsw.gov.au/resources/soc/20110717EstuariesTRS.pdf

Roterman, Y. R., Benayahu, Y., Reshef, L., & Gophna, U. (2015). The gill microbiota of invasive and indigenous Spondylus oysters from the Mediterranean Sea and northern Red Sea. *Environ. Microbiol. Rep., 7*(6), 860-867. doi:10.1111/1758-2229.12315

Roubal, F., Masel, J., & Lester, R. (1989). Studies on *Marteilia sydneyi*, agent of QX disease in the Sydney rock oyster, *Saccostrea commercialis*, with implications for its life cycle. *Mar. Freshwater Res., 40*(2), 155-167. doi:http://dx.doi.org/10.1071/MF9890155

Roughley, T. (1926). *An investigation of the cause of an oyster mortality on the Georges River, New South Wales.* Paper presented at the Linnean Society of New South Wales.

Roughley, T. C. (1922). *Oyster culture on the George's River, New South Wales*. Sydney: Government of New South Wales.

Roughley, T. C. (1933). The Life History of the Australian Oyster (*Ostrea Commercialis*). In *Proceedings of The Linnean Society of New South Wales* (Vol. 58, pp. 279-333). Retrieved from http://biostor.org/reference/68499

Roy, P. S., Williams, R. J., Jones, A. R., Yassini, I., Gibbs, P. J., Coates, B., West, R. J., Scanes, P. R., Hudson, J. P., & Nichol, S. (2001). Structure and function of South-east Australian estuaries. *Estuar. Coast. Shelf S., 53*(3), 351-384. doi:10.1006/ecss.2001.0796

Rua, C. P. J., Trindade-Silva, A. E., Appolinario, L. R., Venas, T. M., Garcia, G. D., Carvalho, L. S., Lima, A., Kruger, R., Pereira, R. C., Berlinck, R. G. S., et al. (2014). Diversity and antimicrobial potential of culturable heterotrophic bacteria associated with the endemic marine sponge *Arenosclera brasiliensis*. *PeerJ, 2*, e419-e419. doi:10.7717/peerj.419

Rubio, A., Frances, J., Coad, P., Stubbs, J., & Guise, K. (2013). The onset and termination of the Qx disease window of infection in Sydney rock oyster (*Saccostrea glomerata*) cultivated in the Hawkesbury river, NSW, Australia. *J. Shellfish Res., 32*(2), 483-496. doi:10.2983/035.032.0228

Salling, H. K., & Bang-Christensen, S. R. (2016). Multi-primer qPCR assay capable of highly efficient and specific detection of the vast majority of all known *Mycoplasma*. *Biologicals, 44*(3), 129-138. doi:https://doi.org/10.1016/j.biologicals.2016.03.003

Sande, M. G., Çaykara, T., Silva, C. J., & Rodrigues, L. R. (2020). New solutions to capture and enrich bacteria from complex samples. *Med. Microbiol. Immun., 209*(3), 335-341. doi:10.1007/s00430-020-00659-1

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., & Garcia, C. (2010). A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. *Microb. Ecol., 59*(4), 787-798. doi:10.1007/s00248-009-9620-y

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., et al. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol., 75*(23), 7537-7541. doi:10.1128/AEM.01541-09

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., Perez, T., Rodrigo, A., Schupp, P. J., Vacelet, J., et al. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J., 6*(3), 564-576. doi:10.1038/ismej.2011.116

Schrobback, P., Pascoe, S., & Coglan, L. (2014). History, status and future of Australia's native Sydney rock oyster industry. *Aquat. Living Resour., 27*(3-4), 153-165. doi:10.1051/alr/2014011

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host Microbe, 12*. doi:10.1016/j.chom.2012.08.003

Shade, A., & Handelsman, J. (2012). Beyond the venn diagram: the hunt for a core microbiome. *Environ. Microbiol., 14*(1), 4-12. doi:10.1111/j.1462-2920.2011.02585.x

Sharp, K. H., Pratte, Z. A., Kerwin, A. H., Rotjan, R. D., & Stewart, F. J. (2017). Season, but not symbiont state, drives microbiome structure in the temperate coral *Astrangia poculata*. *Microbiome, 5*(1), 120. doi:10.1186/s40168-017-0329-8

Shnit-Orland, M., & Kushmaro, A. (2009). Coral mucus-associated bacteria: a possible first line of defense. *FEMS Microbiol. Ecol., 67*(3), 371-380. doi:10.1111/j.1574-6941.2008.00644.x

Shumway, S. E. (1996). Natural Environmental Factors. In V. S. Kennedy, R. I. E. Newell, & A. F. Eble (Eds.), *The Eastern Oyster, Crassostrea virginica* (pp. 467-503): Maryland Sea Grant College, College Park.

Simonian, M., Nair, S. V., Nell, J. A., & Raftos, D. A. (2009). Proteomic clues to the identification of QX disease-resistance biomarkers in selectively bred Sydney rock oysters, *Saccostrea glomerata*. *J. Proteomics, 73*(2), 209-217.

Smith, J. D., & Longmore, A. R. (1980). Behaviour of phosphate in estuarine water. *Nature, 287*(5782), 532-534. doi:10.1038/287532a0

Smith, C. J., & Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol. Ecol., 67*(1), 6-20. doi:10.1111/j.1574-6941.2008.00629.x

Söderhäll, K., & Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol., 10*(1), 23-28. doi:https://doi.org/10.1016/S0952-7915(98)80026-5

Spiers, Z. B., Gabor, M., Fell, S. A., Carnegie, R. B., Dove, M., O'Connor, W., Frances, J., Go, J., Marsh, I. B., & Jenkins, C. (2014). Longitudinal study of winter mortality disease in Sydney rock oysters *Saccostrea glomerata*. *Dis. Aquat. Organ., 110*(1), 151-164.

Stier, T., Drent, J., & Thieltges, D. W. (2015). Trematode infections reduce clearance rates and condition in blue mussels *Mytilus edulis*. *Mar. Ecol. Prog. Ser., 529*, 137-144. Retrieved from https://www.int-res.com/abstracts/meps/v529/p137-144/

Storey, M. A., Andreassend, S. K., Bracegirdle, J., Brown, A., Keyzers, R. A., Ackerley, D. F., Northcote, P. T., & Owen, J. G. (2020). Metagenomic Exploration of the Marine Sponge *Mycale hentscheli* Uncovers Multiple Polyketide-Producing Bacterial Symbionts. *mBio, 11*(2), e02997-02919. doi:10.1128/mBio.02997-19

Strathdee, F., & Free, A. (2013). Denaturing gradient gel electrophoresis (DGGE). *Methods Mol. Biol., 1054*, 145-157. doi:10.1007/978-1-62703-565-1_9

Su, G., Morris, J. H., Demchak, B., & Bader, G. D. (2014). Biological network exploration with Cytoscape 3. *Curr. Protoc. Bioinformatics, 47*, 8.13.11-18.13.24. doi:10.1002/0471250953.bi0813s47

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., & Muroga, K. (1998). *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. *Dis. Aquat. Organ., 33*(2), 111-118. doi:10.3354/dao033111

Thomson, J. M. (1954). The Genera of Oysters and the Australian Species. *Mar. Freshwater Res., 5*(1), 132-168. Retrieved from https://doi.org/10.1071/MF9540132

Tipton, L., Darcy, J. L., & Hynson, N. A. (2019). A Developing Symbiosis: Enabling Cross-Talk Between Ecologists and Microbiome Scientists. *Front. Microbiol., 10*(292). doi:10.3389/fmicb.2019.00292

Trabal, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Asencio-Valle, F., Morales-Bojórquez, E., & Romero, J. (2012). Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. *Microb. Ecol., 64*(2), 555-569. doi:10.1007/s00248-012-0039-5

Travers, M.-A., Boettcher Miller, K., Roque, A., & Friedman, C. S. (2015). Bacterial diseases in marine bivalves. *J. Invertebr. Pathol., 131*, 11-31. doi:10.1016/j.jip.2015.07.010

Tubiash, H. S. (1975). Bacterial pathogens associated with cultured bivalve mollusk larvae. In W. L. Smith & M. H. Chanley (Eds.), *Culture of Marine Invertebrate Animals: Proceedings — 1st Conference on Culture of Marine Invertebrate Animals Greenport* (pp. 61-71). Boston, MA: Springer US.

Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating deep phylogenetic relationships among Cyanobacteria and Plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol., 46*(4), 327-338. doi:10.1111/j.1550-7408.1999.tb04612.x

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., & Knight, R. (2012). Defining the human microbiome. *Nutr. Rev., 70 Suppl 1*(Suppl 1), S38-S44. doi:10.1111/j.1753-4887.2012.00493.x

van de Water, J. A. J. M., Voolstra, C. R., Rottier, C., Cocito, S., Peirano, A., Allemand, D., & Ferrier-Pagès, C. (2018). Seasonal stability in the microbiomes of temperate gorgonians and the Red coral *Corallium rubrum* across the Mediterranean sea. *Microb. Ecol., 75*(1), 274-288. doi:10.1007/s00248-017-1006-y

Vasconcelos, G. J., & Lee, J. S. (1972). Microbial flora of Pacific oysters (*Crassostrea gigas*) subjected to ultraviolet-irradiated seawater. *Appl. Microbiol., 23*(1), 11-16. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC380269/

Volokhov, D. V., Graham, L. J., Brorson, K. A., & Chizhikov, V. E. (2011). Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. *Mol. Cell. Probes, 25*(2), 69-77. doi:https://doi.org/10.1016/j.mcp.2011.01.002

Wallace, R. K., Waters, P., & Rikard, F. S. (2008). *Oyster hatchery techniques*: Southern Regional Aquaculture Center.

Wegner, K. M., Volkenborn, N., Peter, H., & Eiler, A. (2013). Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC microbiol., 13*(1), 252.

Wendling, C. C., Batista, F. M., & Wegner, K. M. (2014). Persistence, seasonal dynamics and pathogenic potential of *Vibrio* communities from Pacific oyster hemolymph. *PloS One, 9*(4), e94256.

Wesche, S. (1995). Outbreaks of *Marteilia sydneyi* in Sidney rock oysters and their relationship with environmental pH. *Bull. Eur. Assoc. Fish Pathol., 15*(1), 23-27.

Wesche, S., Adlard, R., & Lester, R. (1999). Survival of spores of the oyster pathogen *Marteilia sydneyi* (Protozoa, Paramyxea) as assessed using fluorogenic dyes. *Dis. Aquat. Organ., 36*(3), 221-226.

Wilson, S. P., & Hyne, R. V. (1997). Toxicity of Acid-Sulfate Soil Leachate and Aluminum to Embryos of the Sydney Rock Oyster. *Ecotoxicol. Environ. Saf., 37*(1), 30-36. doi:http://dx.doi.org/10.1006/eesa.1996.1514

Wolf, P. H. (1967). Winter mortality hits state's output. *The Fisherman, 2*(9), 20-22.

Wolf, P. H. (1972). Occurrence of a haplosporidan in sydney rock oysters (*Crassostrea commercialis)* from Moreton Bay, Queensland, Australia. *J. Invertebr. Pathol., 19*(3), 416-417. doi:http://dx.doi.org/10.1016/0022-2011(72)90244-3

Wolf, P. H. (1979). Life cycle and ecology of *Marteilia sydneyi* in the Australian oyster, *Crassostrea commercialis*. *Mar. Fish. Rev., 41*(1-2), 70 - 72.

Yao, Z., Yang, K., Huang, L., Huang, X., Qiuqian, L., Wang, K., & Zhang, D. (2018). Disease outbreak accompanies the dispersive structure of shrimp gut bacterial community with a simple core microbiota. *AMB Express, 8*(1), 120. doi:10.1186/s13568-018-0644-x

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(1), 134. doi:10.1186/1471-2105-13-134

Zhu, Y., Sun, C., Song, Y., Jiang, F., Yin, X., Tang, M., & Ding, H. (2016). The study of the adductor muscle-shell interface structure in three Mollusc species. *Acta Oceanol. Sin., 35*(8), 57-64. doi:10.1007/s13131-016-0878-x

Ziegler, M., Roik, A., Porter, A., Zubier, K., Mudarris, M. S., Ormond, R., & Voolstra, C. R. (2016). Coral microbial community dynamics in response to anthropogenic impacts near a major city in the central Red Sea. *Mar. Pollut. Bull., 105*(2), 629-640. doi:10.1016/j.marpolbul.2015.12.045

Zurel, D., Benayahu, Y., Or, A., Kovacs, A., & Gophna, U. (2011). Composition and dynamics of the gill microbiota of an invasive Indo-Pacific oyster in the eastern Mediterranean Sea. *Environ. Microbiol., 13*(6), 1467-1476. doi:10.1111/j.1462-2920.2011.02448.x

Appendix

Aquaculture 527 (2020) 735472

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

The Sydney rock oyster microbiota is influenced by location, season and genetics

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ARTICLE INFO

Keywords:
Microbiota
Sydney rock oyster
QX disease
16S rRNA
Disease resistance

ABSTRACT

Queensland unknown (QX) disease is a significant cause of economic loss for the Sydney rock oyster (SRO) aquaculture industry. Evidence is emerging that QX disease is multi-factorial in nature, with a number of environmental and host factors contributing to disease dynamics. Efforts to mitigate the impacts of QX disease are primarily focused on breeding for disease resistance however, the mechanisms that drive disease resistance are poorly understood. One potential factor influencing disease resistance is the microbiota. To determine the influence of location, season and disease resistance on the SRO microbiota, we used 16S rRNA (V1 – V3 region) amplicon sequencing. The microbiota of six SRO families with two categorised as QX-resistant and four as QX-susceptible, deployed to two different locations (Port Stephens and Wallis Lake, NSW, Australia) and over two seasons (Austral summer and winter), were characterised. As expected, the SRO microbiota was distinct to the microbial community found in seawater. Further, the SRO microbiota was significantly influenced by location and season, with operational taxonomic units (OTUs) assigned to the *Candidatus Hepatoplasma* and *Endozoicomonas* genera identified as significant drivers of microbiota dissimilarity between locations and seasons. Disease resistance also significantly influenced the SRO microbiota but only at the winter time point which is before the typical QX disease period. Overall, OTUs assigned to the *Mycoplasma*, *Borrelia* and *Endozoicomonas* genera were over-represented in QX-resistant SRO microbiota, whereas members of the *Pseudoalteromonas*, *Vibrio*, and *Candidatus Hepatoplasma* genera were over-represented in QX-sensitive microbiota. These findings confirm the influencing role of location and season on the microbiota structure as evidenced in other molluscan species, but also provide preliminary evidence that the microbiota assemblage before the QX disease period may be important for resistance to disease and may provide new avenues for managing SRO aquaculture in the future.

1. Introduction

The Sydney rock oyster (SRO; *Saccostrea glomerata*) is native to Australia, where it is one of the most intensively cultivated oyster species (O'Connor and Dove, 2009; Schrobback et al., 2014). However, since the mid-1970's production of this species has been impacted by QX-disease, which can recurrently cause up to 90% mortality in affected estuaries (Department of Primary Industries, 2016; Nell, 2007; O'Connor and Dove, 2009; Peters and Raftos, 2003; Schrobback et al., 2014). The aetiological agent for QX disease is a spore-forming protozoan parasite called *Marteilia sydneyi*. This parasite has an infection cycle that typically enters through the palps and gills in summer and ends in the oyster digestion gland, impacting nutrient uptake and ultimately causing starvation and death through autumn and into winter (Kleeman et al., 2002; Nell, 2007; Wolf, 1979).

To mitigate the impacts of QX disease, the New South Wales Department of Primary Industries (NSW DPI) has led a selective breeding program using both mass selection methods and family based breeding that has greatly reduced SRO mortalities, with some families showing 85% survival through one cycle of disease (Dove et al., 2020). There is evidence that increased levels of resistance in some families may be linked to higher activity of phenoloxidase, an enzyme thought to be involved in oyster defence mechanisms (Newton et al., 2004), yet the full mechanism(s) for resistance remain unresolved.

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https://doi.org/10.1016/j.aquaculture.2020.735472
Received 31 January 2020; Received in revised form 11 May 2020
Available online 18 May 2020

The oyster microbiota is emerging as a factor in disease dynamics (King et al., 2019a) and is an unexplored factor in SRO QX disease resistance. The potential protective role of the mollusc microbiota has been characterised previously, with some microbial members providing anti-pathogen activities (Offret et al., 2019; Prado et al., 2009). In other studies, the microbiota appears to contribute to disease dynamics, for the Pacific oyster it has been demonstrated that summer mortality in France is due to a progressive replacement of non-virulent commensal vibrios with pathogenic vibrios indicating that microbiota dysbiosis precedes mortality (Lemire et al., 2015). Similarly, Pacific oyster mortality syndrome is polymicrobial in nature with a recent study showing that the viral Ostreid Herpesvirus 1 (OsHV-1) suppresses Pacific oyster immunity, allowing opportunistic bacterial pathogens such as *Vibrio* species to thrive (de Lorgeril et al., 2018). Interestingly, the microbiota of Pacific oyster families bred for resistance to OsHV-1 were significantly different to their disease-susceptible counterparts and had a significantly reduced abundance of *Vibrio* species (King et al., 2019c). In SROs, only one study has investigated the QX-disease-affected microbiota by comparing the digestive gland of QX-infected and uninfected oysters (Green and Barnes, 2010). In QX-infected oysters, bacterial diversity was substantially reduced, with the microbiota dominated by a *Rickettsiales*-like operational taxonomic unit (OTU).

A first step in understanding the role of a microbiota in disease dynamics is characterising its composition and determining the factors that shape its structure. In previous studies in other oyster species, the oyster microbiota has been shown to be influenced by both environmental and host factors including location, temperature, infection state, season, genetics, life stage and resistance to disease (Green and Barnes, 2010; King et al., 2012; King et al., 2019b; King et al., 2019c; Lokmer and Wegner, 2015; Lokmer et al., 2016a). However, there is a paucity of studies examining the factors that influence the SRO microbiota assemblage. Therefore, to characterise the influence of location, season and disease-resistance (genetics) on the SRO microbiota, six SRO families with varying degrees of resistance to QX disease were deployed into two locations and sampled in the Austral summer and winter. Understanding the mechanism(s) that drive disease-resistance, including the potential contribution of the microbiota to disease, are imperative for the successful and sustainable management of SRO aquaculture.

2. Materials and methods

2.1. Experimental design and sampling

Fourty-four different *Saccostrea glomerata* families from the 2015 year class were deployed in the Port Stephens (32°43′12.81″S 152°03′40.52″E) and Wallis Lake (32°11′21.3″S 152°29′09.7″E) estuaries in NSW, Australia. Wallis Lake is a wave-dominated barrier estuary whereas Port Stephens is a tide-dominated drowned valley estuary (Roy et al., 2001). These estuaries are approximately 70 km apart and are not affected by QX disease. These sites were selected to remove the influence of infection- or disease-state on the microbiota. For this study, six families from the 2015 class were selected according to their predicted level of resistance to QX disease using the Estimated Breeding Values (EBVs), which provides an estimation of how well families will perform for a particular trait and the likelihood of passing those traits to their progeny. As EBV is only a predictor, we selected six different families with a predicted range of QX disease resistance to ensure that we had sufficient oyster numbers for comparing the microbiota of oysters with differing QX disease resistance. Subsequent exposure of these families to QX disease at Lime Kiln Bar in the Georges river (33°59′19″S 151°03′21″E) demonstrated that four of the families exhibited ≤50% survival (characterised as QX-susceptible), while the other two families displayed > 50% survival (QX-resistant; Table 1).

Five oysters per family were collected from each site in the 2017 Austral summer (January) and Austral winter (June), four and nine months after deployment respectively (120 oyster samples in total). Oysters were randomly collected by farmers from cultivation trays, placed into labelled plastic bags, transported to the laboratory on ice (3–4 h) and stored whole in their shell at -80 °C for later processing. Because oyster leases could only be accessed by boat, seawater samples were collected from jetties (piers) approximately 800 m away from the oyster leases. The jetties face the oyster leases and are suspended over water that are a few metres deep ensuring no sediment was suspended from the bottom during collection. Ten litres of surface seawater samples were collected and kept on ice during transport to the laboratory. Triplicate seawater samples of 2000 mL for each sampling time were filtered with Durapore Membrane Filters (0.22 μm pore size) for subsequent microbiota analyses. All filtered samples were frozen in liquid nitrogen upon collection in sterile 5 mL cryotubes and kept at -80 °C prior to analysis.

Table 1
2015 year class Sydney rock oyster average family survival (n = 3, ± SD) following exposure to QX disease at Lime Kiln Bar, Georges river. Oysters were deployed to Lime Kiln Bar on 12 December 2016 and oyster survival was counted on 20 September 2017.

Family line	Average survival (%)
F25	59.67 ± 0.58
F22	55.33 ± 3.06
F18	19.67 ± 3.79
F03	3.33 ± 2.31
F32	2.67 ± 3.06
F37	0.67 ± 1.15

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

Environmental parameters (temperature, oxygen, pH, and conductivity) were measured at jetties adjacent to the oyster leases using a WTW multiprobe meter (Multi 3430, Germany) at the time of oyster sample collections. For nutrient analysis, 50 mL triplicate seawater samples were syringe filtered through a 0.45 μm filter into 50 mL sterile falcon tubes, transported to the laboratory on ice, and frozen at -20 °C. Nutrient analysis (nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) and phosphate (PO_4^{3-})) was performed by Envirolab Services Pty Ltd. (Sydney, New South Wales, Australia). From the 10 L of seawater collected above, triplicate 200 mL aliquots were filtered through glass microfiber filters (0.7 μm pore size) and stored at -80 °C for subsequent chlorophyll-a analyses. Chlorophyll a was analysed based on a spectrophotometric method described previously (Ritchie, 2006).

2.3. DNA extractions and 16S rRNA amplicon sequencing

DNA extractions commenced only after the last sample had been collected and frozen. Samples were randomly thawed in batches of 20 and all samples were processed using a single DNA extraction kit. Thawed oysters 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 Petri dish. Approximately 25–50 mg of adductor muscle tissue was then excised using a sterile scalpel blade and placed into a 1.5 mL Eppendorf tube for subsequent DNA extraction using the Qiagen DNeasy Blood and Tissue DNA extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. Haemolymph is often used to study the oyster microbiota (Lokmer et al., 2016a; Lokmer et al., 2016b) but can be difficult to extract from small oysters and is not possible to extract once oysters have been frozen. To minimise variation, we decided to freeze oysters so they could be later processed together. Therefore, the adductor muscle was selected for microbiota analysis as it contains

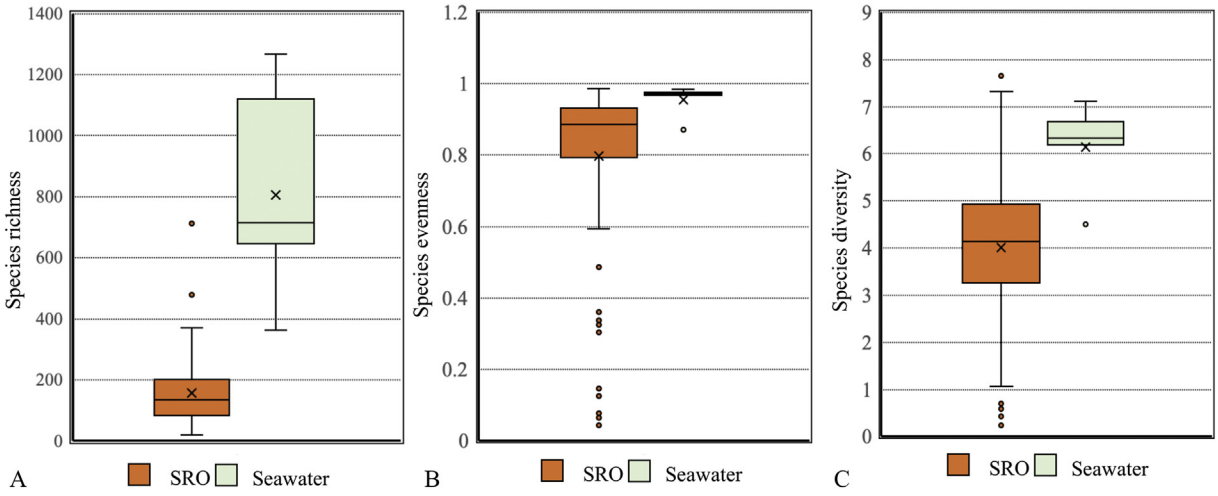

Fig. 1. Box and whisker plot of species richness (A), evenness (B) and diversity (C) for SRO and seawater microbiota. The x in the box plot is the mean of the dataset.

haemolymph sinuses thus allowing us to easily sample the haemolymph. This approach has been successfully used before (King et al., 2019b; King et al., 2019c). The instruments used to process the oysters, including the shucking knife, were cleaned, soaked in 1:15 bleach solution for 15 min and then rinsed with sterile Milli-Q water prior to use and between samples. DNA from filtered seawater samples were extracted using the PowerWater DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocol.

The V1–V3 region of the 16S rRNA gene was amplified by PCR using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′- GWATTACCGCGGCKGCTG-3′) primer pair (Lane, 1991; Turner et al., 1999). The PCR cycling conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30s, 50 °C for 30s and 72 °C for 30s and a final extension of 72 °C for 10 min. Amplicons were sequenced using the Illumina MiSeq platform (2 × 300 bp) 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 (SRP234946) under Bioproject number PRJNA593911.

2.4. Bioinformatics analyses

Demultiplexed paired-end reads were combined using FLASH (Magoč and Salzberg, 2011) and trimmed using Mothur (Schloss et al., 2009) (Parameters: maxhomop = 5, maxambig = 0, minlength = 471, maxlength = 501). Fragments were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and chimeric and singleton sequences were identified and removed using VSEARCH (Rognes et al., 2016). Taxonomic assignment of OTUs were performed in QIIME version 1.9.1 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) against the SILVA v128 dataset (Quast et al., 2013). Mitochondrial and chloroplast data were filtered out of the dataset and the remaining reads were rarefied to the same depth to remove the effect of sampling effort upon analysis. For beta diversity, the relative abundance of OTUs was calculated and all OTUs with a relative abundance below 0.1% were filtered from the dataset. Alpha diversity indices, including species richness (Chao1), species evenness (Simpson) and species diversity (Shannon index) were calculated using QIIME (Caporaso et al., 2010).

2.5. Statistical analyses

Alpha diversity metrics were compared between groups using a Kruskal-Wallis test. All beta diversity analyses were performed with a Bray-Curtis dissimilarity index. To easily visualise how samples related to one another and observe distance matrices between groups, non-metric multidimensional scaling analysis (nMDS) with three dimensions (3D) was used. Patterns elucidated by the 3D nMDS were statistically tested using a permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations using transformed (square root(x)) data. To identify the OTUs driving the difference between the microbial assemblage at different locations or time points, SIMPER analysis was used. All alpha and beta diversity comparisons were performed in the PAST statistical environment (Hammer et al., 2001). To determine whether the relative abundances of OTUs were significantly different between oyster groups with differing QX-resistance, a Welch's *T*-Test was performed using the STAMP (Statistical Analysis of Metagenomic Profiles) software package version 2.1.3 (Parks et al., 2014). A file listing the relative abundance of all OTUs was used as input data along with a metadata file containing location, sampling time and QX-resistance group information. A Welch's T-Test with a p-value of < 0.05 as a statistical cut-off was used. To visualise the significant difference in the relative abundance of OTUs between the QX-sensitive QX-and resistant groups at a single location at each sampling time, extended error bar plots with corrected p-values were produced.

3. Results

Following amplicon sequencing of the 132 samples (oysters and seawater), data were rarefied to 7178 reads retaining a total of 753,690 reads from 105 samples (Supplementary Table 1). After data filtering, a total of 1889 OTUs were observed across the entire dataset. Of these, 1619 and 190 OTUs were unique to the oyster and seawater microbiota respectively, with only 80 OTUs found in both the oyster and seawater samples.

3.1. The SRO microbiota is distinct from the seawater microbiota

Across the entire dataset, species richness, evenness and diversity were higher in seawater samples relative to the SRO adductor muscle microbiota (Fig. 1 and Supplementary Table 2). When grouping all SRO or seawater samples, a 3D nMDS analysis revealed that the composition of the SRO and seawater microbiota were distinct from one another (Supplementary Fig. 1), with these differences confirmed as significantly different by PERMANOVA ($F = 13.54$, $p = .0001$). SIMPER analysis revealed a 99.1% dissimilarity between the SRO and seawater microbiota, with *Candidatus Hepatoplasma* genus (OTU 14887) and *Endozoicomonas* genus (OTU 3829) over-represented in SRO microbiota and driving 5.7% and 2.9% of the difference respectively (Fig. 2 and Supplementary Table 3). In seawater, the *Candidatus Actinomarina*

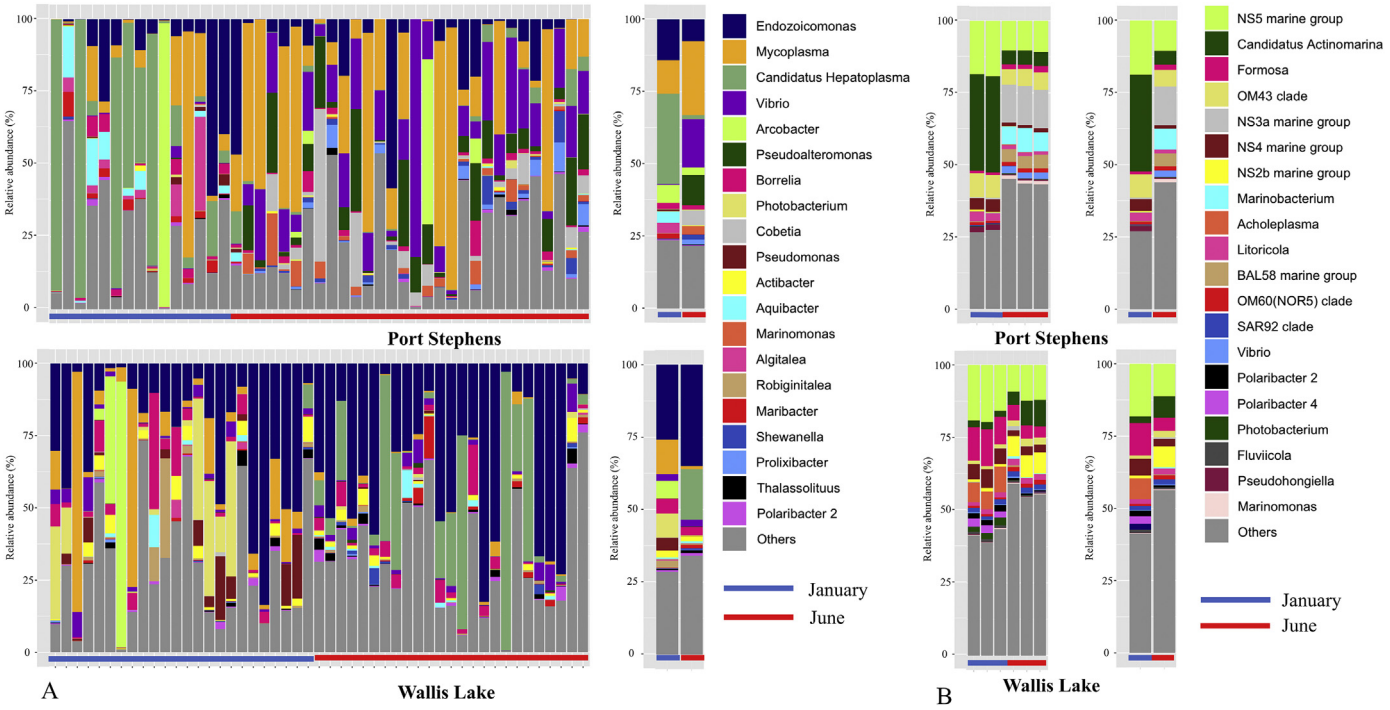

Fig. 2. Microbiota composition of SRO (A) and seawater samples (B) in Port Stephens (upper panels) and Wallis Lake (lower panels) showing the top 20 dominant and remaining taxa in January (underlined by blue bar) and June (underlined by red bar). The right bars in each panel show the mean abundance of each taxon within each group. Data is summarised at the genus level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genus (OTU 22961) and NS5 marine group genus (OTU 5409) were over-represented, driving 4.2% and 3.6% of the difference respectively (Fig. 2 and Supplementary Table 3).

3.2. Location is a factor shaping the SRO microbiota

Overall, Port Stephens had higher temperatures, pH and chlorophyll *a* at each time point, whereas Wallis Lake had higher levels of dissolved oxygen relative to Port Stephens. A rainfall event occurred during the June (winter) sampling at Port Stephens which likely explains the decrease in conductivity and increase in nutrients during this time point (Table 2).

When the total SRO microbiota deployed in Port Stephens and Wallis Lake were compared, species richness and diversity were statistically higher in Wallis Lake ($p = .029$ and $p = .007$ respectively, Supplementary Fig. 2A and Supplementary Table 4). However, no statistical difference in alpha indices was observed when SRO microbiota from Port Stephens and Wallis Lake were independently compared in January and June (Supplementary Fig. 2B and Supplementary Table 4).

Despite some overlap, a 3D nMDS plot showed that SRO microbiota clustered according to location (Fig. 3A) and were significantly different according to site (PERMANOVA, $F = 8.955$, $p = .0001$). This effect of location was also evident within each season in January (Fig. 3B and C; PERMANOVA, $F = 5.117$, $p = .0001$) and June (PERMANOVA, $F = 11.81$, $p = .0001$). Across the entire dataset, the SRO microbiota at Port Stephens and Wallis Lake were 90.5% dissimilar to one another. Similarly, in January and June, the SRO microbiota from the two sites were 90.3% and 91.9% dissimilar respectively. Interestingly, the main dissimilarity contributor, *Candidatus Hepatoplasma* genus (OTU 14887), was over-represented at Port Stephens in January contributing 17.7% to the dissimilarity between microbiota however, was over-represented at Wallis Lake in June contributing 9.6% of the microbiota dissimilarity (Supplementary Table 5). Additionally, a member of the *Endozoicomonas* genus (OTU 1831) was over-represented in Wallis Lake in both January and June contributing 3.0% and 6.4% respectively.

3.3. Season is a factor shaping the SRO microbiota

We next examined whether seasonality influenced the SRO microbiota within a given location. There were no statistical differences in alpha diversity in either Port Stephens or Wallis Lake (Supplementary Fig. 2C and Supplementary Table 4). However, 3D nMDS plots revealed the SRO microbiota at both sites tended to cluster according to sampling time (Fig. 4). This seasonal variability was more pronounced in Port Stephens (PERMANOVA, $F = 10.42$, $p = .0001$) than Wallis Lake (PERMANOVA, $F = 3.451$, $p = .0001$). At Wallis Lake, the SRO microbiota was 86.5% dissimilar with OTUs assigned as members of the *Endozoicomonas* genus (OTU 1831) and the *Candidatus Hepatoplasma* genus (OTU 14887) over-represented in January and June respectively, contributing 8.1% and 10.4% to the microbiota dissimilarity (Supplementary Table 6). At Port Stephens, there was 92.7% dissimilarity in SRO microbiota composition between seasons, with an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) over-represented in January and contributing 16.8% to the dissimilarity. In June, OTUs assigned as *Vibrio* (OTU 2), *Mycoplasma* (OTU 14900) and *Pseudoalteromonas* (OTU 8917) were over-represented, contributing 6.6%, 5.6% and 5.0% to the dissimilarity between seasons respectively (Supplementary Table 6).

3.4. The effect of QX-resistance on the SRO microbiota

Across times and sites, we analysed differences in the oyster microbiota between SROs with different levels of resistance to QX disease. Families were grouped as QX-sensitive if survival was ≤50% and QX-resistant if displayed > 50% survival (Table 1). Species richness was higher in the QX-sensitive group at Port Stephens in January (Average: 74 ± 3.26 vs 143.38 ± 77.87, $p = .039$; Supplementary Table 7). No other significant differences in alpha diversity indices were observed between the QX groups in each location at each time point

Table 2
Environmental parameters in Port Stephens and Wallis Lake at time of sampling.

Time	Temperature (°C)	pH	DO (mg/L)	Conductivity (μS/cm)	NO_3 (mg/L)	NO_2^- (mg/L)	NH_3 (mg/L)	PO_4^{3-} (mg/L)	Chlorophyll a (μg/ml)	Rainfall[a]
Port Stephens										
January	27.8	8.0	8.18	53.3	< 0.005	0.004 ± 0.0	0.012 ± 0.003	0.014 ± 0.003	11.41 ± 1.48	Rainfall 2 days before sampling (0.4 mm). Monthly total rainfall was 69.9 mm
June	24	8.3	8.88	27.6	0.047 ± 0.01	< 0.005	0.038 ± 0.001	< 0.005	23.03 ± 3.13	Rainfall over 6 days including during sampling (average 23.65 mm/day). Monthly total rainfall was 315.1 mm
Wallis Lake										
January	24	7.2	9.5	53.9	< 0.005	0.004 ± 0.0	0.013 ± 0.004	0.007 ± 0.001	9.05 ± 0.62	Rainfall event 2 days before sampling (2.0 mm). Monthly total rainfall was 89.2 mm
June	18.3	8.2	9.07	53.6	0.014 ± 0.014	< 0.005	0.018 ± 0.001	< 0.005	9.52 ± 0.57	Rainfall over 3 days before sampling (average 5.6 mm/day). Monthly total rainfall was 188.1 mm

[a] Data obtained from (Bureau of Meteorology, 2019).

(Supplementary Table 7). PERMANOVA showed statistically significant differences in the microbiota structure of different QX-resistance groups only in June at both locations (Table 3).

At Port Stephens in June, SIMPER analysis revealed a 75.7% dissimilarity between the QX-sensitive and QX-resistant groups with two OTUs (OTU 12669 and OTU 14900) from the *Mycoplasma* genus over-represented in the QX-resistant group and contributing 9.6% and 9.2% to the microbiota dissimilarity. OTUs belonging to the *Pseudoalteromonas* (OTU 8917) and *Vibrio* (OTU 2) genera were over-represented in the QX-sensitive group contributing 6.4% and 6.1% to the microbiota dissimilarity (Supplementary Table 8), while another OTU assigned to the *Vibrio* genus (OTU 1) was over-represented in the QX-resistant microbiota contributing 5.6% dissimilarity (Supplementary Table 8). Additionally, two *Mycoplasma* OTUs (OTU 12669 and OTU 14900) were over-represented in the QX-resistant group, contributing 9.6% and 9.2% to the microbiota dissimilarity. At Wallis Lake in June, SIMPER revealed 77.9% microbiota dissimilarity between the QX groups. A member assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) was over-represented in the QX-sensitive group and contributed 15.86% of the microbiota dissimilarity, whereas 5 OTUs, all assigned to the *Endozoicomonas* genus (OTUs 1831, 3829, 6283, 3483 and 4530), were over-represented in the QX-resistant microbiota.

To further decipher beta diversity patterns between QX-resistant and -sensitive SRO's, STAMP with a Welch's *T*-Test was used. This analysis identified members of the *Vibrio* (OTU 2, $p = .003$) and *Colwellia* (OTU 3670, $p = .028$) genera with significantly higher relative abundance in the QX-sensitive group from Port Stephens in June (Fig. 5A). In Wallis Lake, a member assigned as the *Thiohalocapsa* genus (OTU 11899) had a significantly higher relative abundance in QX-sensitive oysters ($p = .025$), whereas OTUs assigned to the *Borrelia* (OTU 651, $p = .038$) and *Endozoicomonas* (OTU 4530, $p = .047$) genera had a significantly higher relative abundance in QX-resistant oysters (Fig. 5B).

4. Discussion

This study investigated the influence of location, season and oyster genetics (QX-resistance) on shaping the SRO microbiota. Despite the filter-feeding nature of oysters, our results indicate that the SRO microbiota is highly distinct from the planktonic microbiota within the surrounding seawater. It is possible that part of the observed variation is due to the seawater samples being collected from jetties 800 m from the oyster leases however, it is unlikely that the main bacterial patterns in the seawater would substantially vary across this small distance. Additionally, it is also possible that a part of the observed variation is due to the use of different DNA extraction kits for the oysters and water samples. Nevertheless, the patterns we observed are consistent with previous studies on the microbiota of the Pacific oyster (Lokmer et al., 2016a; Lokmer et al., 2016b).

The microbiota varies between oyster tissues (King et al., 2012; King et al., 2020; Lokmer et al., 2016b) however, some overlap is observed such as the genus *Mycoplasma* which is dominant in the adductor muscle, gill, stomach, digestive gland and haemolymph (Green and Barnes, 2010; King et al., 2012; King et al., 2019b; King et al., 2020; Wegner et al., 2013). Here, we elected to use the adductor muscle as it allows sampling of the circulatory haemolymph from the sinuses. Overall, the SRO microbiota was dominated by OTUs assigned to the *Candidatus Hepatoplasma*, *Endozoicomonas* and *Mycoplasma* genera. *Candidatus Hepatoplasma* has been found associated with various marine organisms such as starfish (Nakagawa et al., 2017), Norway lobsters (Meziti et al., 2012), corals (van de Water et al., 2018) and starlet sea anemones (Mortzfeld et al., 2016). However, the function of this bacterium in marine organisms, including SROs, is unknown. *Mycoplasma* is consistently identified in healthy oysters including Eastern oysters, Pacific oyster and SROs (Green and Barnes, 2010; King et al.,

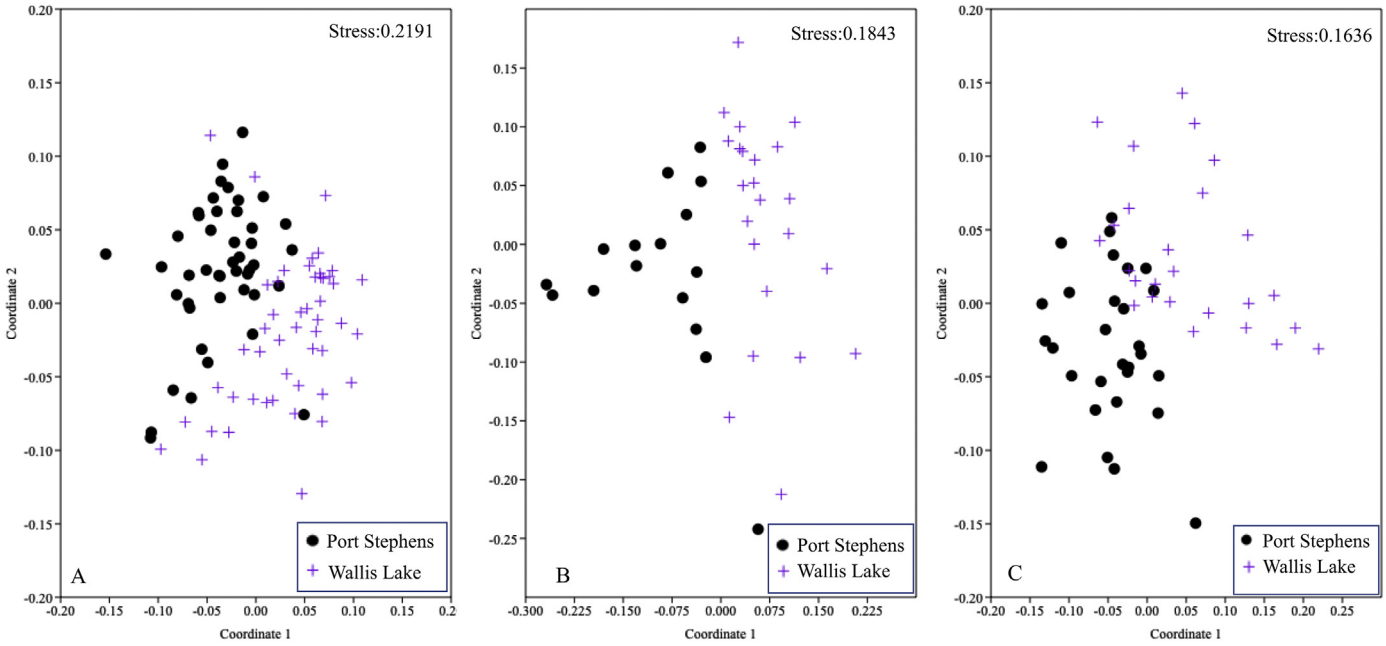

Fig. 3. 3D nMDS plots of total SRO microbiota (A) and those from January (B) and June (C) show separation according to location.

2012; King et al., 2019b; King et al., 2019c; Wegner et al., 2013) suggesting that these bacteria are potentially important for oyster health. Members of the *Endozoicomonas* genus have been found to be associated with numerous marine organisms (Neave et al., 2016) such as sponges (Nishijima et al., 2013; Rua et al., 2014) and corals (Bayer et al., 2013; Ziegler et al., 2016) with members of this genus previously shown to comprise a large proportion of the Indo-Pacific (Roterman et al., 2015; Zurel et al., 2011) and Black-Lipped pearl oyster (Dubé et al., 2019) bacterial communities. In sponges and corals, these bacteria play a role in nitrogen and carbon recycling, provision of proteins to their hosts and production of antibiotics (Neave et al., 2017; Nishijima et al., 2013;

Table 3
PERMANOVA results comparing the microbiota of QX-sensitive (F03, F18, F32 and F37) and QX-resistant (F022 and F025) families at each location and time point.

	Port Stephens	Wallis Lake
January	$F = 1.184$, $p = .2233$	$F = 1.1$, $p = .263$
June	$F = 1.562$, $p = .0491$	$F = 1.614$, $p = .0378$

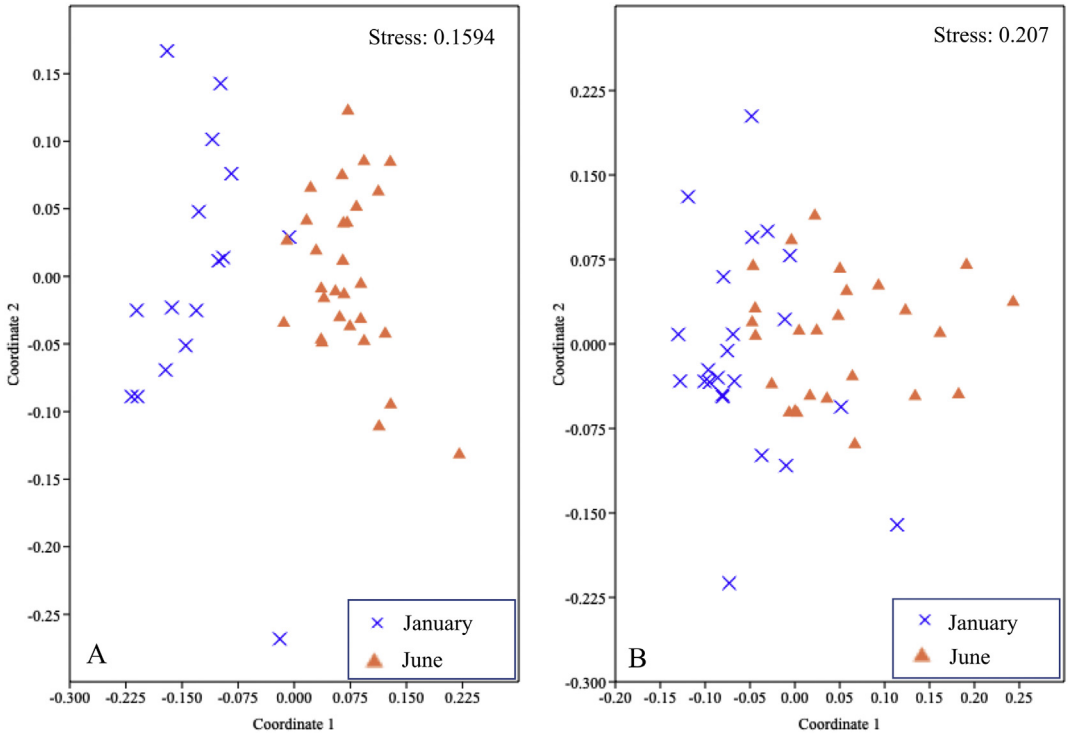

Fig. 4. 3D nMDS plots of SRO microbiota in Port Stephens (A) and Wallis Lake (B) separating according to time of sampling.

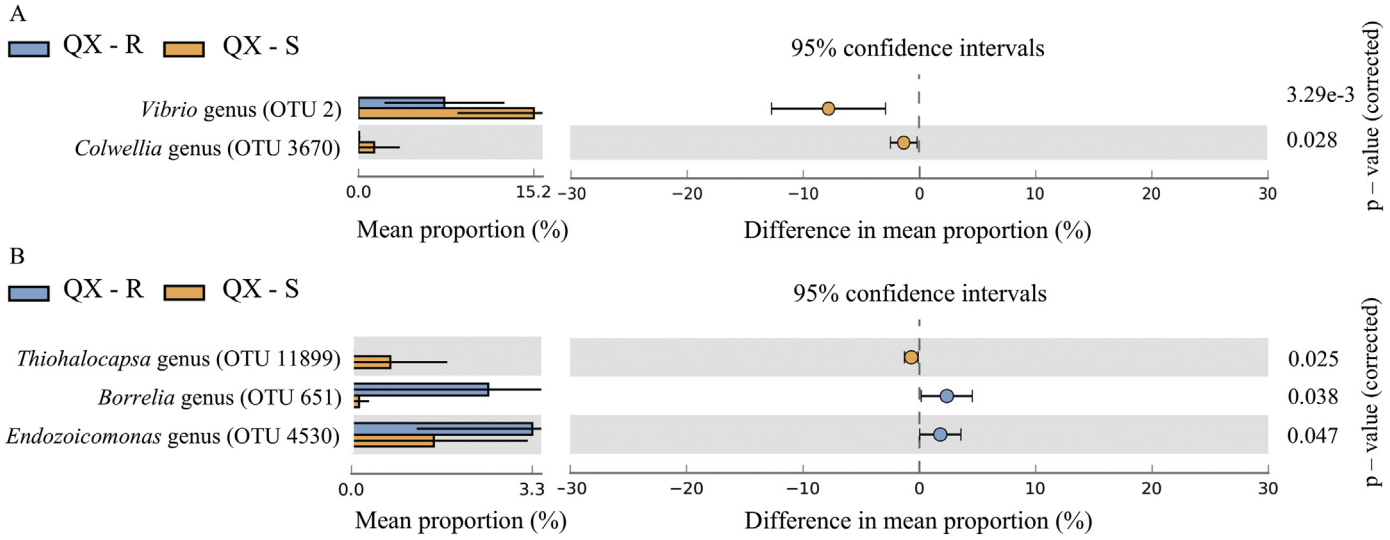

Fig. 5. Extended error bar plots showing OTUs with a significant difference in relative abundance between the QX-sensitive (QX - S) and resistant groups (QX - R) at Port Stephens (A) and Wallis Lake (B) in June.

Rua et al., 2014) and may suggest a similar role in SROs.

4.1. The SRO microbiota is influenced by location

The same oyster families were deployed in Port Stephens and Wallis Lake reducing the influence of genetics as a confounding factor in our analyses and allowing us to investigate whether location or season influence the composition of the SRO microbiota. Consistent with previous studies that have characterised the influence of location on the oyster microbiota (King et al., 2012; Ossai et al., 2017; Roterman et al., 2015; Trabal et al., 2012; Zurel et al., 2011), we observed that SRO microbiota was significantly different between two sites which are approximately 70 km apart and differ in estuarine type (Roy et al., 2001). Data collected in this study identified higher chlorophyll *a* concentrations and temperature in Port Stephens relative to Wallis Lake. While both estuaries have similar percentages of agricultural land usage in their respective catchments (approximately 30%), Port Stephens has significantly higher sediment and nutrient inputs compared to Wallis Lake (Roper et al., 2011). Given the higher nutrient and sediment loads at Port Stephens, these factors could explain the microbiota variability between the locations. A member of the *Endozoicomonas* genus (OTU 1831) was more abundant in Wallis Lake than in Port Stephens at both sampling times. In coral species, the anthropogenically influenced coral microbiota (*Pocillopora verrucosa* and *Acropora hemprichii*) was marked by a reduction of *Endozoicomonas* relative abundance (Ziegler et al., 2016), suggesting that the lower relative abundance of this bacteria in SROs at Port Stephens could be related to the higher nutrient and sediment loads.

4.2. The SRO microbiota is influenced by season

In a number of marine organisms, including corals (Sharp et al., 2017) and Pacific oysters (Pierce et al., 2016; Zurel et al., 2011), there is evidence for significant temporal heterogeneity in microbiota composition. Consistent with these findings, we observed a significant influence of season (summer versus winter) on the SRO microbiota for both locations. At Port Stephens, seasonal shifts in environmental conditions were dominated by changing temperature, chlorophyll *a* and conductivity, while at Wallis Lake, seasonal changes in environmental parameters were mostly driven by temperature and pH. Previous studies have characterised the influence of temperature on the oyster microbiota (Lokmer and Wegner, 2015; Pierce et al., 2016) and salinity perturbations have also been observed to influence the oyster microbiota (del Refugio Castañeda Chávez et al., 2005; Larsen et al., 2013). Seasonal shifts in the SRO microbiota were characterised by changes in the relative abundance of several OTUs, including those assigned to the *Candidatus Hepatoplasma* and *Vibrio* genera. Interestingly, we observed inverse patterns for the relative abundance of an OTU assigned to the *Candidatus Hepatoplasma* genus (OTU 14887) between the two sampling sites. At Port Stephens, this OTU was significantly more abundant in summer, while at Wallis Lake, it was considerably more abundant in winter. The environmental data collected at the time suggests no similarities between the Port Stephens summer and Wallis Lake winter samples that could explain this pattern (conductivity was similar for these two sampling points but conductivity did not change between the Wallis Lake summer and winter sampling points) and this OTU was rare or absent in the seawater communities, therefore future studies should increase the suite of environmental parameters collected to explain these patterns. At both locations, a member of the *Vibrio* genus (OTU 2) had a higher relative abundance in winter than in summer. This pattern is interesting given that *Vibrio* typically exhibit preferences for warm water temperatures. However, some *Vibrio* species such as *Vibrio splendidus,* have elsewhere been found to be most abundant during winter and spring (Arias et al., 1999; Pujalte et al., 1999). It is also conceivable that other environmental factors, such as chlorophyll *a* or nutrient levels, underpinned the higher winter relative abundance of this *Vibrio* species (OTU 2).

4.3. The SRO microbiota is influenced by disease resistance

Oyster genetics have previously been shown to influence the Pacific oyster microbiota structure (King et al., 2019c; Wegner et al., 2013), with the microbiota of disease-resistant Pacific oysters showing a significantly different structure to disease-susceptible oysters (King et al., 2019c). However, the influence of genetics on the Pacific oyster microbiota can be superseded by stress, such as temperature perturbations (Wegner et al., 2013). In this study, we observed significant differences of the microbiota between QX-resistant and QX-susceptible oysters, but only in winter (June). This pattern suggests that there is a synergistic interaction of genetics and environmental drivers in shaping the SRO microbiota, which is consistent with previous studies in marine organisms such as Pacific oysters (Wegner et al., 2013) and corals (Klaus et al., 2005). While QX disease typically occurs between November to May (Bezemer et al., 2006; Rubio et al., 2013), infections by *M. sydneyi* that cause no mortality (Adlard and Wesche, 2005) have been observed between May to July (Rubio et al., 2013), corresponding to the period

where microbiota heterogeneity between resistance groups was observed in this study. This could indicate that the microbiota assemblage prior to the peak mortality period is important and could contribute to QX disease dynamics, although future studies should consider performing a temporal study to capture possible microbiota dynamics.

A previous study characterising the influence of disease-resistance on Pacific oyster microbiota identified disease-susceptible oysters as having a higher absolute abundance of *Vibrio* species (King et al., 2019c). Interestingly, this pattern is consistent with observations made in this study, where at Port Stephens we observed an over-representation of an OTU assigned to the *Vibrio* genus (OTU 2) in QX-susceptible oysters. *Vibrio* species are commonly implicated as pathogens affecting marine molluscs such as clams, mussels and oysters (Paillard et al., 2004; Travers et al., 2015). For example, *Vibrio* species have a crucial role in summer mortalities of Pacific oysters (de Lorgeril et al., 2018; Garnier et al., 2007; King et al., 2019b; Lemire et al., 2015; Petton et al., 2015; Saulnier et al., 2010; Sugumar et al., 1998) with a non-virulent *Vibrio* community replaced by a pathogenic one (Lemire et al., 2015). Given their role in marine molluscs and other oyster diseases, investigating whether *Vibrio* species influence QX-disease dynamics would be of interest. At Wallis Lake, an OTU assigned to the *Endozoicomonas* genus (OTU 4530) was significantly over-represented in the QX-resistant oysters. *Endozoicomonas* bacteria have found to be associated with many marine organisms such as sponges, corals and oysters (Dubé et al., 2019; Neave et al., 2016; Roterman et al., 2015; Zurel et al., 2011). Given the importance of *Endozoicomonas* species in sponges and corals (Neave et al., 2017; Nishijima et al., 2013; Rua et al., 2014), future studies should investigate their potential role in QX-resistant oysters.

5. Conclusion

There is emerging evidence that the microbiota of benthic organisms, including oysters, are dynamic and driven by multiple factors, but the impact of location, season and genetics (disease resistance) on the SRO microbiota have not been reported previously. Understanding the factors that drive SRO microbiota composition are pivotal when deciphering the role of the microbiota during disease events, and to explain microbiota shifts prior to, or during, disease. However, this is currently hindered by a paucity of SRO microbiota studies. This study demonstrated that the SRO microbiota assemblage is influenced by location and season, which highlights the importance of performing temporal studies at individual locations as interpreting microbiota patterns from other locations or time points can lead to erroneous microbiota explanations. Further, breeding for QX disease resistance (genetics) was found to influence the SRO microbiota although this was only observed in the winter. This sampling time point is before the typical QX disease period, which may indicate that a microbiota shift could be a factor in QX disease dynamics. Overall, these data suggest that there is a synergistic interaction of genetics and environmental drivers in shaping the SRO microbiota.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by an Australian Research Council Linkage Project (LP160101785), a Cooperative Research Centre Project (CRC-P 2016-805; Future Oysters), led by the Australian Seafood Industry Pty Ltd. in partnership with a number of Australian research organisations and, Ausgem, a research partnership initiated between the University of Technology Sydney and the New South Wales Department of Primary Industries. VKN was supported by a University of Technology Sydney – Vietnam International Education Development (UTS - VIED) Scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735472.

References

Adlard, R.D., Wesche, S., 2005. Aquatic Animal Health Subprogram: Development of a Disease Zoning Policy for Marteilia Sydneyi to Support Sustainable Production, Health Certification and Trade in the Sydney Rock Oyster. Retrieved from. http://www.frdc.com.au/Archived-Reports/FRDC%20Projects/2001-214-DLD.pdf.

Arias, C.R., Macian, M.C., Aznar, R., Garay, E., Pujalte, M.J., 1999. Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. J. Appl. Microbiol. 86 (1), 125–134. https://doi.org/10.1046/j.1365-2672.1999.00641.x.

Bayer, T., Neave, M.J., Alsheikh-Hussain, A., Aranda, M., Yum, L.K., Mincer, T., Hughen, K., Apprill, A., Voolstra, C.R., 2013. The microbiome of the Red Sea coral *Stylophora pistillata* is dominated by tissue-associated *Endozoicomonas* bacteria. Appl. Environ. Microbiol. 79 (15), 4759. https://doi.org/10.1128/AEM.00695-13.

Bezemer, B., Butt, D., Nell, J., Adlard, R., Raftos, D., 2006. Breeding for QX disease resistance negatively selects one form of the defensive enzyme, phenoloxidase, in Sydney rock oysters. Fish Shellfish Immunol. 20 (4), 627–636. https://doi.org/10.1016/j.fsi.2005.08.007.

Bureau of Meteorology, 2019. Daily Rainfall. Climate Data Online. Retrieved from. http://www.bom.gov.au/climate/data/index.shtml. http://www.bom.gov.au/climate/data/index.shtml.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336. https://doi.org/10.1038/nmeth.f.303.

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., et al., 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. Nat. Commun. 9 (1), 4215. https://doi.org/10.1038/s41467-018-06659-3.

del Refugio Castañeda Chávez, M., Sedas, V.P., Orrantia Borunda, E., Reynoso, F.L., 2005. Influence of water temperature and salinity on seasonal occurrences of *Vibrio cholerae* and enteric bacteria in oyster-producing areas of Veracruz, México. Mar. Pollut. Bull. 50 (12), 1641–1648. https://doi.org/10.1016/j.marpolbul.2005.06.036.

Department of Primary Industries, 2016. NSW Oyster Industry Sustainable Aquaculture Strategy. Department of Primary Industries, NSW Retrieved from. https://www.dpi.nsw.gov.au/_data/assets/pdf_file/0006/638250/NSW-oyster-industry-sustainable-aquaculture-strategy-2016.pdf.

Dove, M., Kube, P., Wilkie, E., Cumbo, V., Raftos, D., O'Connor, W., New Soth Wales Department of Primery Industries (Fisheries), 2020. Accelerated Sydney rock oyster (SRO) breeding research. In: Future Oysters CRC-P Project 2016–802, (Retrieved from Taylors Beach).

Dubé, C.E., Ky, C.-L., Planes, S., 2019. Microbiome of the black-lipped pearl oyster *Pinctada margaritifera*, a multi-tissue description with functional profiling. Front. Microbiol. 10 (1548). https://doi.org/10.3389/fmicb.2019.01548.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26 (19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J.-L., 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb. Ecol. 53 (2), 187–196. https://doi.org/10.1007/s00248-006-9061-9.

Green, T.J., Barnes, A.C., 2010. Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, Marteilia sydneyi. J. Appl. Microbiol. 109 (2), 613–622. https://doi.org/10.1111/j.1365-2672.2010.04687.x.

Hammer, Øyvind, Harper David, A.T., Paul, D.R., 2001. Past: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4 (1), 9. Retrieved from. https://palaeo-electronica.org/2001_1/past/past.pdf.

King, G.M., Judd, C., Kuske, C.R., Smith, C., 2012. Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. PLoS One 7 (12), e51475. https://doi.org/10.1371/journal.pone.0051475.

King, W.L., Jenkins, C., Seymour, J.R., Labbate, M., 2019a. Oyster disease in a changing environment: decrypting the link between pathogen, microbiome and environment. Mar. Environ. Res. 143, 124–140. https://doi.org/10.1016/j.marenvres.2018.11.007.

King, W.L., Jenkins, C., Go, J., Siboni, N., Seymour, J.R., Labbate, M., 2019b. Characterisation of the Pacific oyster microbiome during a summer mortality event. Microb. Ecol. 77 (2), 502–512. https://doi.org/10.1007/s00248-018-1226-9.

King, W.L., Siboni, N., Williams, N.L.R., Kahlke, T., Nguyen, K.V., Jenkins, C., Dove, M., O'Connor, W., Seymour, J.R., Labbate, M., 2019c. Variability in the composition of Pacific oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 µvar disease. Front. Microbiol. 10, 473. https://doi.org/10.

3389/fmicb.2019.00473.
King, W.L., Siboni, N., Kahlke, T., Dove, M., O'Connor, W., Mahbub, K.R., Jenkins, C., Seymour, J.R., Labbate, M., 2020. Regional and oyster microenvironmental scale heterogeneity in the Pacific oyster bacterial community. FEMS Microbiol. Ecol. https://doi.org/10.1093/femsec/fiaa054.
Klaus, J.S., Frias-Lopez, J., Bonheyo, G.T., Heikoop, J.M., Fouke, B.W., 2005. Bacterial communities inhabiting the healthy tissues of two Caribbean reef corals: interspecific and spatial variation. Coral Reefs 24 (1), 129–137. https://doi.org/10.1007/s00338-004-0447-1.
Kleeman, S.N., Adlard, R.D., Lester, R.J.G., 2002. Detection of the initial infective stages of the protozoan parasite *Marteilia sydneyi* in *Saccostrea glomerata* and their development through to sporogenesis. Int. J. Parasitol. 32 (6), 767–784. https://doi.org/10.1016/S0020-7519(02)00025-5.
Lane, D.J., 1991. 16S/23S rRNA Sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons Ltd., West Sussex, pp. 115–147.
Larsen, A.M., Scott Rikard, F., Walton, W.C., Arias, C.R., 2013. Effective reduction of *Vibrio vulnificus* in the eastern oyster (*Crassostrea virginica*) using high salinity depuration. Food Microbiol. 34 (1), 118–122. https://doi.org/10.1016/j.fm.2012.11.009.
Lemire, A., Goudenege, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., Le Roux, F., 2015. Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. ISME J. 9 (7), 1523–1531. https://doi.org/10.1038/ismej.2014.233.
Lokmer, A., Wegner, M., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J. 9 (3), 670–682. https://doi.org/10.1038/ismej.2014.160.
Lokmer, A., Goedknegt, M.A., Thieltges, D.W., Fiorentino, D., Kuenzel, S., Baines, J.F., Wegner, K.M., 2016a. Spatial and temporal dynamics of Pacific oyster hemolymph microbiota across multiple scales. Front. Microbiol. 7, 1367. https://doi.org/10.3389/fmicb.2016.01367.
Lokmer, A., Kuenzel, S., Baines, J.F., Wegner, K.M., 2016b. The role of tissue-specific microbiota in initial establishment success of Pacific oysters. Environ. Microbiol. 18 (3), 970–987. https://doi.org/10.1111/1462-2920.13163.
Magoč, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27 (21), 2957–2963. https://doi.org/10.1093/bioinformatics/btr507.
Meziti, A., Mente, E., Kormas, K.A., 2012. Gut bacteria associated with different diets in reared *Nephrops norvegicus*. Syst. Appl. Microbiol. 35 (7), 473–482. https://doi.org/10.1016/j.syapm.2012.07.004.
Mortzfeld, B.M., Urbanski, S., Reitzel, A.M., Künzel, S., Technau, U., Fraune, S., 2016. Response of bacterial colonization in *Nematostella vectensis* to development, environment and biogeography. Environ. Microbiol. 18 (6), 1764–1781. https://doi.org/10.1111/1462-2920.12926.
Nakagawa, S., Saito, H., Tame, A., Hirai, M., Yamaguchi, H., Sunata, T., Aida, M., Muto, H., Sawayama, S., Takaki, Y., 2017. Microbiota in the coelomic fluid of two common coastal starfish species and characterization of an abundant *Helicobacter*-related taxon. Sci. Rep. 7, 8764. https://doi.org/10.1038/s41598-017-09355-2.
Neave, M.J., Apprill, A., Ferrier-Pagès, C., Voolstra, C.R., 2016. Diversity and function of prevalent symbiotic marine bacteria in the genus *Endozoicomonas*. Appl. Microbiol. Biotechnol. 100 (19), 8315–8324. https://doi.org/10.1007/s00253-016-7777-0.
Neave, M.J., Michell, C.T., Apprill, A., Voolstra, C.R., 2017. *Endozoicomonas* genomes reveal functional adaptation and plasticity in bacterial strains symbiotically associated with diverse marine hosts. Sci. Rep. 7, 40579. https://doi.org/10.1038/srep40579.
Nell, J., 2007. Diseases of Sydney Rock Oysters. NSW Department of Primary Industries, NSW, Australia Retrieved from. https://marine-aquaculture.extension.org/wp-content/uploads/2019/05/Diseases-of-Sydney-Rock-Oysters.pdf.
Newton, K., Peters, R., Raftos, D., 2004. Phenoloxidase and QX disease resistance in Sydney rock oysters (*Saccostrea glomerata*). Dev. Comp. Immunol. 28 (6), 565–569. https://doi.org/10.1016/j.dci.2003.10.004.
Nishijima, M., Adachi, K., Katsuta, A., Shizuri, Y., Yamasato, K., 2013. Endozoicomonas numazuensis sp. nov., a gammaproteobacterium isolated from marine sponges, and emended description of the genus Endozoicomonas Kurahashi and Yokota 2007. Int. J. Syst. Evol. Microbiol. 63 (Pt 2), 709–714. https://doi.org/10.1099/ijs.0.042077-0.
O'Connor, W., Dove, M., 2009. The changing face of oyster culture in New South Wales, Australia. J. Shellfish Res. 28, 803–811.
Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., Le Chevalier, P., Fleury, Y., 2019. Protective efficacy of a *Pseudoalteromonas* strain in European abalone, *Haliotis tuberculata*, infected with *Vibrio harveyi* ORM4. Probiotics Antimicro. 11 (1), 239–247. https://doi.org/10.1007/s12602-018-9389-8.
Ossai, S., Ramachandran, P., Ottesen, A., Reed, E., DePaola, A., Parveen, S., 2017. Microbiomes of American oysters (*Crassostrea virginica*) harvested from two sites in the Chesapeake bay. Genome Announ. 5 (30). https://doi.org/10.1128/genomeA.00729-17. (e00729–00717).
Paillard, C., Le Roux, F., Borrego, J.J., 2004. Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. Aquat. Living Resour. 17 (4), 477–498.
Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30 (21), 3123–3124. https://doi.org/10.1093/bioinformatics/btu494.
Peters, R., Raftos, D.A., 2003. The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Aquaculture 223 (1), 29–39. https://doi.org/10.1016/S0044-8486(03)00169-8.
Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., Le Roux, F., 2015. *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. Front. Microbiol. 6, 686. https://doi.org/10.3389/fmicb.2015.00686.
Pierce, M.L., Ward, J.E., Holohan, B.A., Zhao, X., Hicks, R.E., 2016. The influence of site and season on the gut and pallial fluid microbial communities of the eastern oyster, *Crassostrea virginica* (Bivalvia, Ostreidae): community-level physiological profiling and genetic structure. Hydrobiologia 765 (1), 97–113.
Prado, S., Montes, J., Romalde, J.L., Barja, J.L., 2009. Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. Int. Microbiol. 12 (2), 107–114.
Pujalte, M., Ortigosa, M., Macián, M., Garay, E., 1999. Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int. Microbiol. 2 (4), 259–266.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219.
Ritchie, R.J., 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth. Res. 89 (1), 27–41. https://doi.org/10.1007/s11120-006-9065-9.
Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584. https://doi.org/10.7717/peerj.2584.
Roper, T., Creese, B., Scanes, P., Stephens, K., Williams, R., Dela-Cruz, J., Coade, G., Coates, B., Fraser, M., 2011. Assessing the Condition of Estuaries and Coastal Lake Ecosystems in NSW. Retrieved from. https://www.environment.nsw.gov.au/resources/soc/20110717EstuariesTRS.pdf.
Roterman, Y.R., Benayahu, Y., Reshef, L., Gophna, U., 2015. The gill microbiota of invasive and indigenous Spondylus oysters from the Mediterranean Sea and northern Red Sea. Environ. Microbiol. Rep. 7 (6), 860–867. https://doi.org/10.1111/1758-2229.12315.
Roy, P.S., Williams, R.J., Jones, A.R., Yassini, I., Gibbs, P.J., Coates, B., West, R.J., Scanes, P.R., Hudson, J.P., Nichol, S., 2001. Structure and function of south-east Australian estuaries. Estuar. Coast. Shelf Sci. 53 (3), 351–384. https://doi.org/10.1006/ecss.2001.0796.
Rua, C.P.J., Trindade-Silva, A.E., Appolinario, L.R., Venas, T.M., Garcia, G.D., Carvalho, L.S., Lima, A., Kruger, R., Pereira, R.C., Berlinck, R.G.S., et al., 2014. Diversity and antimicrobial potential of culturable heterotrophic bacteria associated with the endemic marine sponge *Arenosclera brasiliensis*. PeerJ 2, e419. https://doi.org/10.7717/peerj.419.
Rubio, A., Frances, J., Coad, P., Stubbs, J., Guise, K., 2013. The onset and termination of the Qx disease window of infection in Sydney rock oyster (*Saccostrea glomerata*) cultivated in the Hawkesbury river, NSW, Australia. J. Shellfish Res. 32 (2), 483–496. https://doi.org/10.2983/035.032.0228.
Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., 2010. A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. Microb. Ecol. 59 (4), 787–798. https://doi.org/10.1007/s00248-009-9620-y.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537–7541. https://doi.org/10.1128/AEM.01541-09.
Schrobback, P., Pascoe, S., Coglan, L., 2014. History, status and future of Australia's native Sydney rock oyster industry. Aquat. Living Resour. 27 (3–4), 153–165. https://doi.org/10.1051/alr/2014011.
Sharp, K.H., Pratte, Z.A., Kerwin, A.H., Rotjan, R.D., Stewart, F.J., 2017. Season, but not symbiont state, drives microbiome structure in the temperate coral *Astrangia poculata*. Microbiome 5 (1), 120. https://doi.org/10.1186/s40168-017-0329-8.
Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., Muroga, K., 1998. *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. Dis. Aquat. Org. 33 (2), 111–118. https://doi.org/10.3354/dao033111.
Trabal, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Asencio-Valle, F., Morales-Bojórquez, E., Romero, J., 2012. Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. Microb. Ecol. 64 (2), 555–569. https://doi.org/10.1007/s00248-012-0039-5.
Travers, M.-A., Boettcher Miller, K., Roque, A., Friedman, C.S., 2015. Bacterial diseases in marine bivalves. J. Invertebr. Pathol. 131, 11–31. https://doi.org/10.1016/j.jip.2015.07.010.
Turner, S., Pryer, K.M., Miao, V.P.W., Palmer, J.D., 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. 46 (4), 327–338. https://doi.org/10.1111/j.1550-7408.1999.tb04612.x.
van de Water, J.A.J.M., Voolstra, C.R., Rottier, C., Cocito, S., Peirano, A., Allemand, D., Ferrier-Pagès, C., 2018. Seasonal stability in the microbiomes of temperate gorgonians and the red coral *Corallium rubrum* across the Mediterranean Sea. Microb. Ecol. 75 (1), 274–288. https://doi.org/10.1007/s00248-017-1006-y.
Wegner, K.M., Volkenborn, N., Peter, H., Eiler, A., 2013. Disturbance induced decoupling between host genetics and composition of the associated microbiome. BMC Microbiol. 13 (1), 252.
Wolf, P.H., 1979. Life cycle and ecology of *Marteilia sydneyi* in the Australian oyster, *Crassostrea commercialis*. Mar. Fish. Rev. 41 (1–2), 70–72.
Ziegler, M., Roik, A., Porter, A., Zubier, K., Mudarris, M.S., Ormond, R., Voolstra, C.R., 2016. Coral microbial community dynamics in response to anthropogenic impacts near a major city in the Central Red Sea. Mar. Pollut. Bull. 105 (2), 629–640. https://doi.org/10.1016/j.marpolbul.2015.12.045.
Zurel, D., Benayahu, Y., Or, A., Kovacs, A., Gophna, U., 2011. Composition and dynamics of the gill microbiota of an invasive indo-Pacific oyster in the eastern Mediterranean Sea. Environ. Microbiol. 13 (6), 1467–1476. https://doi.org/10.1111/j.1462-2920.2011.02448.x.