Interrogating the microbiome for improved understanding of Pacific oyster diseases

William L King

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

December 2019

University of Technology Sydney
School of Life Sciences
Climate Change Cluster

2019

Certificate of Original Authorship

I, William King, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Signature: Production Note:
Signature removed prior to publication.

Date: 16/09/19

Table of contents

Acknowledgments

First and foremost, I would like to thank Maurizio Labbate. Including my honours project I have been a student of Maurizio's for nearly five complete years and I really could not have asked for a better supervisor. Maurizio has been absolutely instrumental shaping me into the scientist I am today and without his guidance and support I would not be where I am right now. In fact, without his sassy quirkiness the last few years would have been especially dull. Although I now fear I have picked up a few of his sassy traits. I especially want to point out that Maurizio has always been very welcoming of the occasional unplanned meeting where I was able to just drop by whenever I had a new curious thought. These little meetings have definitely been very important to me not just to improve my scientific learning but by also offering the opportunity to unload some thoughts anytime I needed to talk. These meetings have made me realise the importance of unscheduled meetings and I definitely plan to have the same policy with my own students one day (hopefully).

Next, I would like to thank Justin Seymour. Justin was incredibly patient as I was getting to my feet as I had come from a fairly molecular focused honours project and ventured into the realm of microbial ecology. In addition to Maurizio, Justin has been incredibly supportive over the last four years. He has always nudged me in the right direction and without him I would not have adapted so easily to a new field. One particular thing that Justin has helped me refine is my writing skills. He is an absolute machine when it comes to writing and I have definitely done my best to mould myself to his writing skills to improve my own skillset. Further, our little chats in the gym have been quite important when I needed some unscheduled guidance about navigating my scientific career or when crafting stories for my manuscripts.

Because of these gym chats, Justin's bench press hasn't improved in a few years so I'm sure he is looking forward to me leaving…

Another key figure during my PhD has been Nachshon Siboni. Anyone that knows Nachshon knows that he is legend, he is simply unforgettable! I would like to thank Nachshon for personally teaching me absolutely everything he knows about microbial ecology and I definitely would not be anywhere near as developed as a scientist without his support. Although, I don't think he remembered my name for an entire year! Nachshon's support over the last four years cannot be measured, he has always been available for chats and was always there every single time I needed help. Nachshon is also a really funny guy and can take a joke so our meetings were always full of laughs which always brightened up my days. I will definitely miss having Nachshon around when I inevitably depart on the next stage of my career.

Next, I would like to say a bulk thank you to the Labbate and Seymour laboratory members, those other PhD students that I have interacted with along the way, particularly those in the 'too many flies in the office squad', my family and all of the tech staff. While there are way too many people to individually thank, I would like to specifically thank Rami, Elizabeth, Daniela, Regan and Nathan. Nathan, it has been an absolute joy seeing you develop from the cheeky undergraduate student that I was training during his internship to the independent PhD student you are today. Rami, Elizabeth and Daniela, you've all been supporting me for pretty much my entire PhD project and without our daily gossip sessions I surely would have gotten more work done… kidding! You three, Rami especially, have definitely kept me grounded and chatting to you guys always brightened up my day. I wish you all the best with your future endeavours.

Finally, I would like to thank my partner in crime, Sarah Iwanoczko. Sarah's support during my PhD project has been absolutely invaluable, especially when I have been bogged down by work. Sarah has always helped to keep my chin up and she has nudged me through the harder parts of my PhD. Words can't describe the positive impact Sarah has had on my life and that has definitely reflected on my positive attitude throughout my PhD. No matter what happens in the future, I will always cherish Sarah's support over the last few years and without her my days wouldn't be nearly as bright. I have especially enjoyed our American adventures together and I hope there is many more worldwide adventures to come. You really are amazing Sarah, thank you!

Publications associated with this thesis

Chapter One

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

Chapter Two

King, W.L., Jenkins, C., Go, J., Siboni, N., Seymour, J.R., and Labbate, M. (2019). Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. *Microbial Ecology*. 2019;77(2):502-12. 10.1007/s00248-018-1226-9

Chapter Three

King, W.L., Siboni, N., Williams, N.L.R., Kahlke, T., Nguyen, K.V., Jenkins, C., Dove, M., O'Connor, W., Seymour, J.R., and Labbate, M. (2019). Variability in the composition of Pacific Oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 μvar disease. *Frontiers in Microbiology* 10, 473. 10.3389/fmicb.2019.00473

Supporting Publication One

Green, T.J., Siboni, N., **King, W.L.**, Labbate, M., Seymour, J.R., and Raftos, D. (2018). Simulated Marine Heat Wave Alters Abundance and Structure of *Vibrio* Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event. *Microbial Ecology*. 10.1007/s00248-018-1242-9

Conference presentations associated with this thesis

William L King, Cheryl Jenkins, Jeffrey Go, Nahshon Siboni, Justin R Seymour, Maurizio Labbate. Presentation titled: Microbiome investigations in a Pacific Oyster summer mortality outbreak in Port Stephens, New South Wales, Australia. Australian Microbial Ecology (AusME) meeting.

William L King, Cheryl Jenkins, Jeffrey Go, Justin R Seymour, Maurizio Labbate. Presentation titled: The effect of microbiological and environmental factors on summer mortality events in Pacific oysters. Australian Shellfish Quality Assurance Science Day (ASQAAC).

William L King, Cheryl Jenkins, Jeffrey Go, Nachshon Siboni, Justin R Seymour, Maurizio Labbate. Presentation titled: Characterisation of the pacific oyster microbiome during a summer mortality event. 110th Annual Meeting of the National Shellfisheries Association.

William L King, Nachshon Siboni, Michael Dove, Wayne O'Connor, Cheryl Jenkins, Justin R Seymour, Maurizio Labbate. Presentation titled: Polymicrobial involvement in OsHV-1 outbreaks (and other diseases). Oysters Australia Research and Development Meeting

William L King, Cheryl Jenkins, Jeffrey Go, Nachshon Siboni, Justin R Seymour, Maurizio Labbate. Presentation titled: Elucidating links between the Pacific Oyster microbiome and summer mortality events. 4th FRDC Australasian Scientific Conference.

Maurizio Labbate, **William L King**, Khue Viet Nguyen, Nachshon Siboni, Michael Dove, Wayne O'Connor, Cheryl Jenkins, Justin R Seymour.

Presentation titled: Is there a role for microbiomes in oyster mortality events? Australian Shellfish Quality Assurance Science Day (ASQAAC).

William L King, Nachshon Siboni, Cheryl Jenkins, Jeffrey Go, Nathan Williams, Tim Kahlke, Michael Dove, Wayne O'Connor, Maurizio Labbate, Justin R Seymour. Presentation titled: Insights into the influence of the pacific oyster microbiome in oyster disease and resistance. 17th International Symposium on Microbial Ecology, International Society for Microbial Ecology (ISME).

List of figures

Chapter One

Chapter Two

List of tables

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Abbreviations

C. gigas	*Crassostrea gigas*
S. glomerata	*Saccostrea glomerata*
O. edulis	*Ostrea edulis*
C. virginica	*Crassostrea virginica*
ROS	Reactive Oxygen Species
OsHV-1	Ostreid herpes virus 1
OsHV-1 μvar	Ostreid herpes virus 1 microvariant
P. marinus	*Perkinsus marinus*
H. nelsoni	*Haplosporidium nelsoni*
M. sydneyi	*Marteilia sydneyi*
B. roughleyi	*Bonamia roughleyi*
M. refringens	*Marteilia refringens*
B. ostreae	*Bonamia ostreae*
M. mackini	*Mikrocytos mackini*
R. crassostreae	*Roseovarius crassostreae*
N. crassostreae	*Nocardia crassostreae*
MSX	Multinucleate Sphere Unknown X
ROD	Roseovarius Oyster Disease
QX	Queensland Unknown
POMS	Pacific Oyster Mortality Syndrome
V. tubiashii	*Vibrio tubiashii*
V. splendidus	*Vibrio splendidus*
V. alginolyticus	*Vibrio alginolyticus*
V. aestuarianus	*Vibrio aestuarianus*
V. lentus	*Vibrio lentus*
V. harveyi	*Vibrio harveyi*
V. coralliilyticus	*Vibrio coralliilyticus*

V. crassostreae	*Vibrio crassostreae*
V. angillarum	*Vibrio angillarum*
V. diabolicus	*Vibrio diabolicus*
V. mediterranei	*Vibrio mediterranei*
V. azureus	*Vibrio azureus*
V. brasiliensis	*Vibrio brasiliensis*
V. chagasii	*Vibrio chagasii*
V. fortis	*Vibrio fortis*
V. vulnificus	*Vibrio vulnificus*
V. campbellii	*Vibrio campbellii*
V. sinaloensis	*Vibrio sinaloensis*
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. rotiferanus	*Vibrio rotiferanus*
ASI	Australian Seafood Industries
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
OTU	Operational Taxonomic Unit
ZOTU	Zero-radius Operational Taxonomic Unit
NSW	New South Wales
DPI	Department of Primary Industries
SRA	Sequence Read Archive
QIIME	Quantitative Insights Into Microbial Ecology
nMDS	Non-metric multidimensional scaling analysis
ANOVA	Analysis of Variance
ANOSIM	Analysis of Similarities
PERMANOVA	Permutational multivariate analysis of variance

CCA	Canonical Correspondence Analysis
SIMPER	Analysis of similarity percentages
PCoA	Principal Coordinates Analysis
RDP	Ribosomal Database Project
GIT	Gastrointestinal Tract
EBV	Estimated Breeding Values
RG	Resistance Group
qPCR	Quantitative Polymerase Chain Reaction
CV	Coefficient of Variation
WGS	Whole Genome Sequencing
FISH	Fluorescence in situ hybridization
MINE	Maximal Information-based Nonparametric Exploration
CR	Clyde River
GR	Georges River
HR	Hawkesbury River
SH	Shoalhaven
PS	Port Stephens
WA	Wapengo
Mt	Mantle
Gl	Gill
Am	Adductor muscle
Dg	Digestive gland
NCBI	National Center for Biotechnology Information
hsp60	Heat shock protein 60
BLAST	Basic Local Alignment Search Tool
NaCl	Sodium Chloride
LB	Lysogeny Broth
nt	Nucleotide

dNTP	Deoxyribonucleotide triphosphate
μL	Microlitre
μM	Micromolar
km	Kilometre
L	Litre
bp	Base pair
mg	Milligrams
ng	Nanograms

Abstract

Oyster aquaculture represents a significant portion of both the Australian, and the global economy, with *Crassostrea gigas* (the Pacific oyster) representing the most heavily cultivated commercial species. However, infectious diseases have emerged as a major obstacle for the successful growth and sustainability of the oyster aquaculture industry. Oyster diseases are often complex, occurring as a result of disturbance in the synergistic relationship between the host, environment, and pathogen/s. Perturbations of environmental factors (e.g. temperature, salinity, nutrients, pH) can have direct influences on the oyster's immune system, and can allow for the proliferation and transmission of oyster pathogens. In particular, two major pathogens of *C. gigas*, ostreid herpesvirus 1 (OsHV-1) and *Vibrio* species, are both strongly driven by temperature. One such understudied factor that may influence oyster disease dynamics is the oyster microbiome. Studies in other model systems have shown the involvement of the microbiome in animal health, disease, and behavior. Because of this, it is likely the oyster microbiome also plays a role in oyster disease dynamics. The work presented in this thesis aimed to use a microbiome approach to provide further understanding of oyster diseases.

Thesis prelude – rationale, significance and aims

The Australian aquaculture industry is valued at $1.31 billion AUD, representing 97,000 tonnes of production (ABARES, 2017). Of this, the oyster aquaculture industry contributes $97 million AUD, and 11, 300 tonnes of production (ABARES, 2017), making it a valuable contributor to the Australian economy. However, a major hurdle to the continued growth and sustainability of the oyster industry are infectious diseases (Lafferty et al., 2015).

Of the commercially cultivated oyster species, *Crassostrea gigas* (the Pacific oyster) is the most heavily cultivated globally (FAO, 2016a). Despite this, commercial cultivation of *C. gigas* has been continually challenged with disease outbreaks facilitated by viral, bacterial and unknown aetiological agents (Lipovsky, 1972; Paillard et al., 2004; Jenkins et al., 2013; King et al., 2019a). Current efforts to mitigate the impact of *C. gigas* diseases are focused on breeding for disease resistance (Dégremont, 2011; Dégremont et al., 2016b). This usually involves exposing oysters to disease in the field and breeding the surviving oysters (Dégremont, 2011). While breeding for disease resistance has been successful in reducing the impact of these diseases, the mechanism/s behind this protection are poorly understood.

Due to the economic importance of *C. gigas* cultivation, studies have sought to examine the causative factors driving these oyster disease outbreaks, with shifts in the environment (perturbations) often implicated as 'triggers' for disease (Burge et al., 2006; Malham et al., 2009; Jenkins et al., 2013; Mortensen et al., 2016; Go et al., 2017). These oyster diseases are complex, often preceding from a disturbance in the synergistic relationship between the host, environment, and pathogen. For example, shifts in environmental

conditions (such as increasing temperature) can drive oyster pathogen transmission and abundance (Petton et al., 2013), while also acting as an immune suppressant to the oyster, as they near their thermal limits (Bougrier et al., 1995). One such host associated factor that may be contributing to oyster disease dynamics is the oyster microbiome.

In recent years, the oyster microbiome has drawn an increasing amount of attention to determine its role in oyster disease dynamics (Lokmer and Wegner, 2015; Petton et al., 2015; Green et al., 2019; King et al., 2019a; King et al., 2019b; King et al., 2019c). However, at the onset of this PhD project, most disease-focused microbiome studies had been culture dependent (Garnier et al., 2007; Petton et al., 2013; Wendling et al., 2014; Lemire et al., 2015; Petton et al., 2015), with only one study employing culture-independent sequencing techniques (Lokmer and Wegner, 2015). Because of this and because of considerable *C. gigas* disease outbreaks in Australia in previous years (Jenkins et al., 2013; Go et al., 2017), this PhD project set out to gain further insight into the oyster microbiome.

This thesis has set out to address five aims, with the overarching goal to provide an improved understanding of oyster diseases using a microbiome approach. Ultimately, the information provided in this thesis will set the framework for future studies by identifying potential probiotic targets in the oyster microbiome and providing new microbiome approaches to oyster diseases for future disease-focused observational studies.

The below aims correspond to chapters one to five accordingly:

Aim one: To provide a critical review of oyster diseases, with an emphasis on the environmental drivers and the potential role of the microbiome.

Aim two: To use a microbiome approach to investigate a *C. gigas* disease outbreak in Port Stephens.

Aim three: To elucidate how breeding *C. gigas* for disease resistance influences microbiome composition and to identify bacteria associated with disease resistance.

Aim four: To determine microbiome patterns across geographic locations and tissue-types and identify core taxa innately tied to the *C. gigas* microbiome.

Aim five: To develop an amplicon sequencing assay for improved taxonomic resolution of the *Vibrio* community and to apply it to a laboratory *C. gigas* disease event.

Chapter One

Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment

Chapter One - 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 marine environmental research.
- Permission is not required by the publisher for inclusion of this publication in this thesis for non-commercial purposes.

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

Publication status: Published

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

Date: 16/09/19

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

1.1 Abstract

Shifting environmental conditions are known to be important triggers of oyster diseases. The mechanism(s) behind these synergistic effects (interplay between host, environment and pathogen/s) are often not clear, although there is evidence that shifts in environmental conditions can affect oyster immunity and, pathogen growth and virulence. However, the impact of shifting environmental parameters on the oyster microbiome and how this affects oyster health and susceptibility to infectious pathogens remains understudied. In this review, we summarise the major diseases afflicting oysters with a focus on the role of environmental factors that can catalyse or amplify disease outbreaks. We also consider the potential role of the oyster microbiome in buffering or augmenting oyster disease outbreaks and suggest that a deeper understanding of the oyster microbiome, its links to the environment and its effect on oyster health and disease susceptibility, is required to develop new frameworks for the prevention and management of oyster diseases.

1.2 Introduction

Oysters are filter-feeding bivalve molluscs that inhabit estuarine and coastal environments. They encompass a number of different species, many of which are heavily farmed for human consumption, supporting valuable aquaculture industries. In 2005, global bivalve aquaculture was responsible for 13.6 million metric tons of production, valued at $1.82 billion USD, with oysters responsible for 4.8 million metric tons of production (Pawiro, 2010). Four oyster species, namely, *Crassostrea gigas* (the Pacific oyster), *Saccostrea glomerata* (formerly *S. commercialis* and also known as the Sydney rock oyster), *Ostrea edulis* (the European flat oyster) and *Crassostrea virginica* (the Eastern oyster or American cupped oyster) are

amongst the most heavily cultivated historically and/or currently across different regions of the world.

Infectious diseases have become a major obstacle for the successful growth and sustainability of oyster aquaculture industries, with a range of diseases having severe detrimental effects on oyster yields. For example, historical outbreaks of *C. virginica* diseases contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). While diseases of *S. glomerata* in Australia, and *O. edulis* in Europe, have also severely diminished their production capacity (Robert et al., 2013; Schrobback et al., 2015; FAO, 2016c). Another species of oyster, *Crassostrea angulata*, was extensively cultivated in France prior to the 1970's before the industry was completely wiped out as a consequence of infectious disease outbreaks, resulting in this species being replaced by imported *C. gigas* (Roch, 1999). These few examples highlight just some of the impacts that infectious diseases have had on global oyster cultivation.

Since oysters are typically reared in uncontrolled and often dynamic coastal and estuarine environments, it is often difficult to predict, manage and control infectious disease outbreaks. Management strategies designed to control the spread of pathogens are further constrained by the ability of marine pathogens to rapidly spread over large distances, due to reduced dispersion barriers in aquatic habitats, relative to terrestrial environments (McCallum et al., 2003). Increasing evidence is showing that oyster diseases have strong environmental drivers such as temperature. Notably, outbreaks are often more severe closer to the tropics (Leung and Bates, 2013) likely due to the preference of many pathogens to grow in warmer waters (Leung and Bates, 2013) or the exertion of temperature stress as oysters reach their thermal limits (Bougrier et al., 1995). Within the context of temperature

driven disease outbreaks, the implications of climate change (i.e. warming waters in non-tropical areas) on pathogen spread, transmission and virulence are a concern for future food security (Harvell et al., 2002). Specific examples supporting this concern include warming oceans driving the geographic spread of *Perkinus marinus*, the parasite responsible for dermo disease in *C. virginica* (Ford, 1996; Cook et al., 1998) and, the enhanced replication and transmission of the *C. gigas* disease-causing herpesvirus OsHV-1 and growth of *Vibrio* species in *C. gigas* tissues at warmer temperatures (Petton et al., 2013; Renault et al., 2014).

The disease process has traditionally been viewed as a 'one pathogen one disease' system, a classical view pioneered by Robert Koch now known as Koch's postulates (Koch, 1884; Löffler, 1884). Since that time, our understanding of infectious disease processes has evolved from a 'classical view' to one of an 'ecological view', in which multiple factors contribute to or amplify the disease process (Wilson, 1995). As with most infectious processes, many oyster diseases appear to be complex and often proceed as a result of a shift or fracture in the interplay between environmental (e.g. temperature, salinity, pH, nutrients) and biological factors, including oyster fitness, the oyster microbiome, the abundance and virulence of external pathogens and their potential vectors (e.g. phytoplankton). Detangling the causative mechanisms of disease from this complex "interactome" (the suite of biotic and abiotic factors that participate in disease processes) is not trivial – in particular, little information is known regarding the role of the microbiome in disease protection or susceptibility. In order to develop more effective strategies for managing infectious outbreaks within oyster harvesting practices, a new understanding of the interactome and the role of the microbiome is necessary. In this review, the major diseases affecting oyster aquaculture will be covered and in particular, the potential synergistic

importance of the oyster microbiome and local environmental parameters in these infectious outbreaks will be evaluated.

1.3 The oyster life cycle, anatomy and distribution

In this section, we will focus on four major commercial oyster species, including *C. gigas*, *S. glomerata*, *O. edulis* and *C. virginica*, which are harvested in a number of regions across the globe **(**Figure 1.1). *C. gigas* is the most widely grown species, with commercial industries in the USA, Canada, Mexico, Chile, Argentina, South Africa, Namibia, China, Japan, Australia and a number of European countries, in particular France (FAO, 2016a). *C. virginica* is grown exclusively in the USA, Canada and Mexico (FAO, 2016b), while *S. glomerata* is only grown in Australia (FAO, 2016d). The limited production of *O. edulis* is restricted to several European nations, the USA and South Africa (FAO, 2016c).

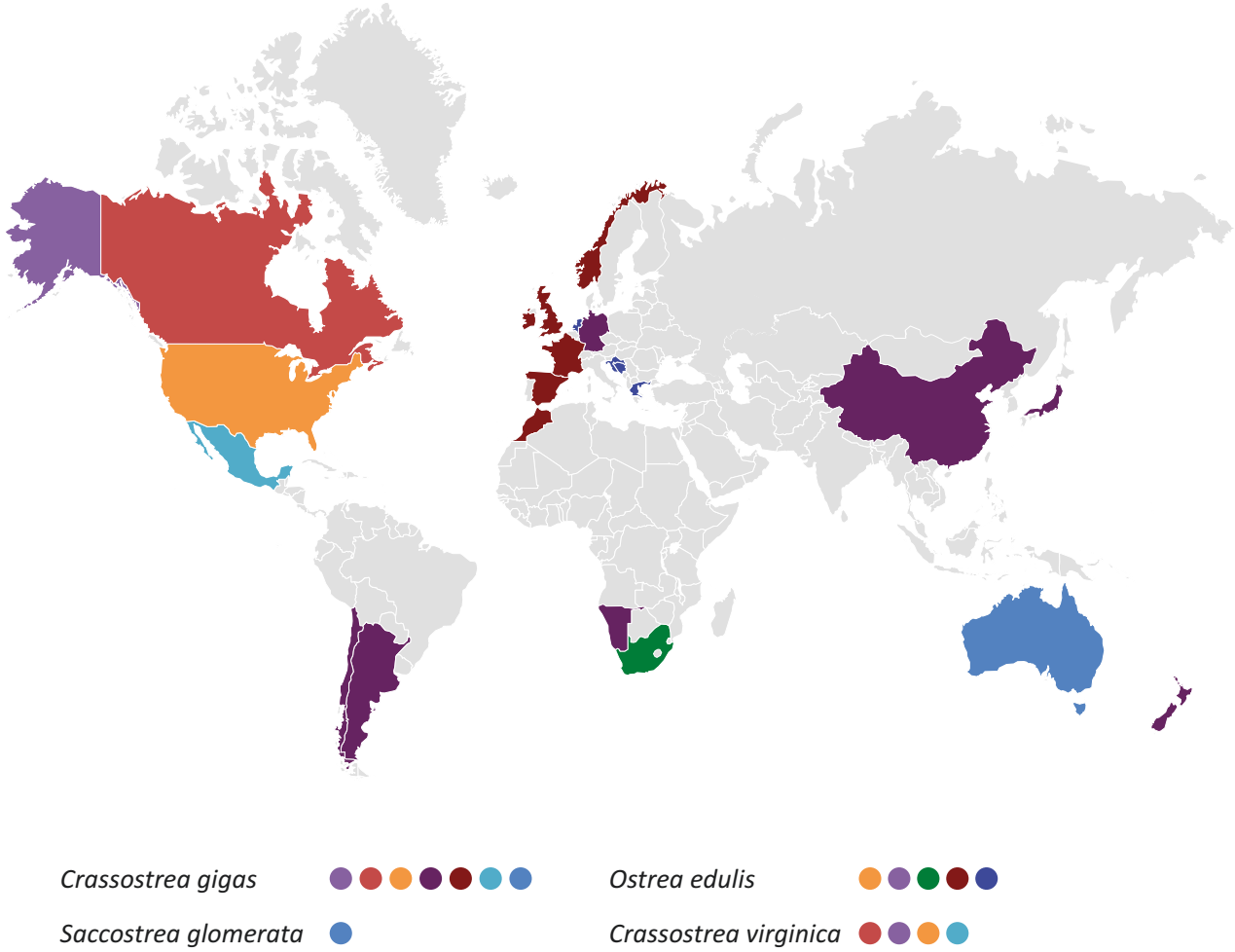


Figure 1.1: Global cultivation of four oyster species. *C. gigas* is grown in the largest number of countries, spanning North and South America, Western Europe and Australia. While *S. glomerata* is only grown in Australia. *C. virginica* is exclusively grown in North America, whereas *O. edulis* is grown in the USA, a number of European countries and South Africa.

There are numerous microbial and viral diseases that can infect one or more stages of the oyster life cycle. Across all species of oysters, the general oyster life cycle is relatively consistent (Figure 1.2). The life cycle begins with spawning, which is dependent on temperature and location (Fujiya, 1970; Wallace, 2001; FAO, 2016a; d; c). Following spawning events, fertilisation occurs, resulting in the development of a free-swimming planktonic larva (trochophore) (Wallace, 2001). At this stage, the oyster larvae are particularly vulnerable to infection by mostly viral and bacterial pathogens (Hine et al., 1992; Luna-González et al., 2002; Elston et al., 2008). After settlement on a hard surface, metamorphosis occurs developing into a juvenile oyster form called spat (Wallace, 2001). Similar to the larval form, spat are prone to infection by bacterial and viral pathogens (Waechter et al., 2002; Friedman et al., 2005). After 12-40 months of growth, the spat grows into a commercially harvestable adult oyster. Relative to the earlier forms, adult oysters are more resistant to viral infection (Dégremont, 2013) with infections from protozoan parasites more likely (Friedman and Perkins, 1994; Green and Barnes, 2010).

The oyster possesses a number of specialised tissues and organs to help it survive in its environment (Figure 1.2). The gills draw in water and directs the collected food particles (such as phytoplankton) to the palps, which sort the food particles before they enter the digestive system. The digestive gland is a common site for protozoan parasite infection often culminating in oyster starvation (Alderman, 1979; Ewart and Ford, 1993; Kleeman et al., 2002). The mantle acts as a sensory organ to initiate opening and closing of the shell, and forms the oyster's shell (Quayle, 1988; FAO, 2016e). Shell infections are observed from some bacterial species, resulting in mantle lesions and abnormal shell deposits (Bricelj et al., 1992). The heart is responsible for circulating the oyster hemolymph, a clear fluid that acts as

the oyster ‘blood’ and contains cells called hemocytes with immune functions (Bachere, 1991). Previous research has indicated that viral pathogens are able to invade and replicate within these hemocytes (Morga et al., 2017). Finally, the gonad represents the reproductive system, which involves the production and release of gametes (spawning) (FAO, 2016e).

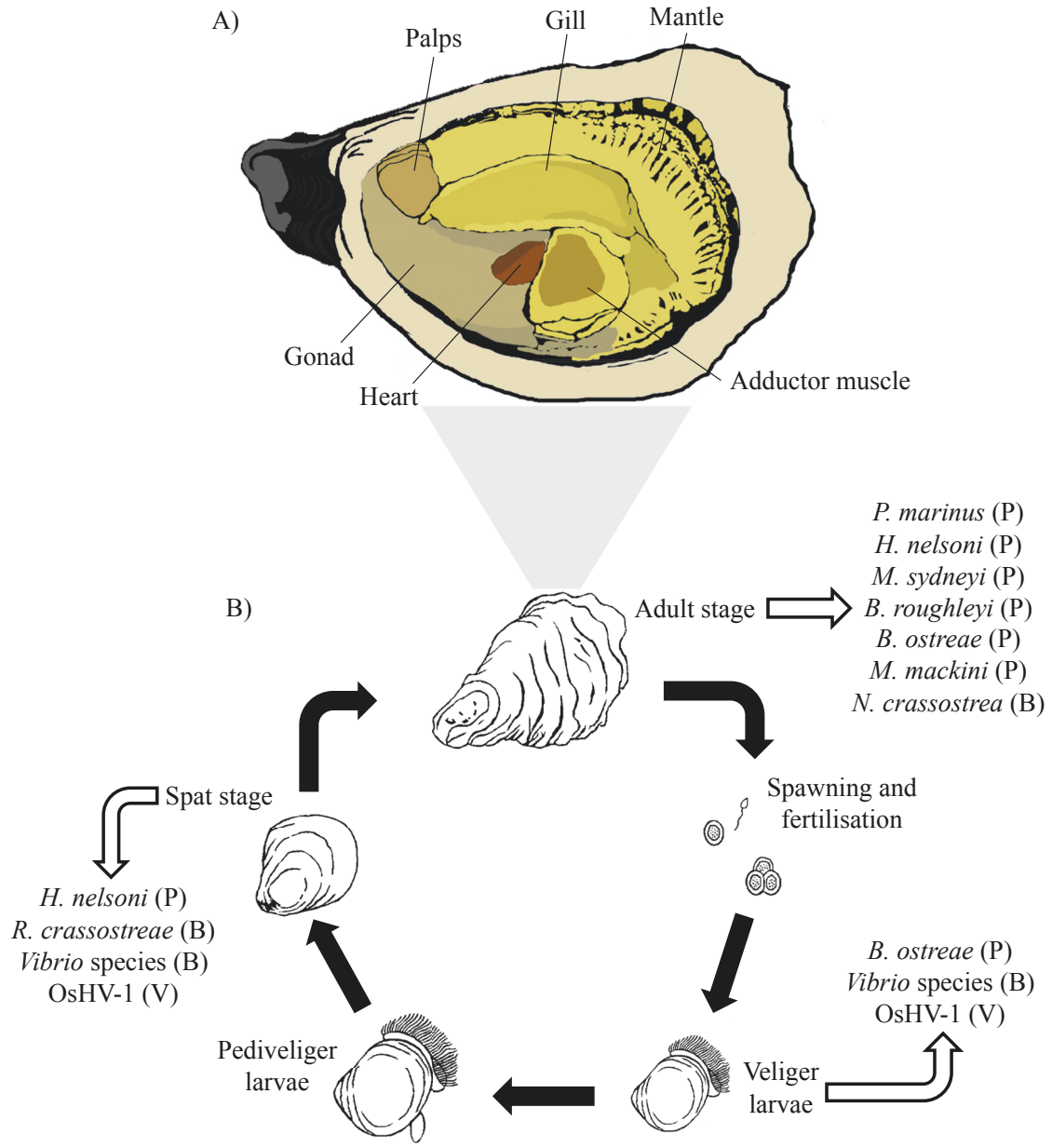


Figure 1.2: The basic anatomy A) and generalised life cycle of oysters B). Oyster pathogens infect various stages of the oyster life cycle. Bacterial and viral pathogens typically infect the spat and larval stages, while the protozoan parasites dominantly infect the adult stages. Black arrows depict the life cycle progression. Black hollow arrows highlight the known pathogens of commercial oysters at each life stage. (P), (B), and (V) represent parasites, bacteria, and viral agents respectively. Image produced by Sarah J Iwanoczko.

1.4 Oyster immunology

Oysters are filter feeders, filtering around 163 litres per day (Riisgård, 1988) and given that the average litre of seawater contains more than a billion microbes, oysters are constantly exposed to a large number of microorganisms present in seawater. In order to combat pathogenic microorganisms, the innate immune system of the oyster is its primary defence (Schmitt et al., 2012a). This immunity is primarily facilitated by hemocytes (Figure 1.3), and molecules/proteins contained in both the hemolymph and epithelial mucus secretions (Cheng and Rodrick, 1975; Itoh and Takahashi, 2008; Pales Espinosa et al., 2014; Allam and Pales Espinosa, 2016).

The oyster hemolymph is not sterile, with low concentrations (10^2-10^5 cells mL^{-1}) of bacteria, primarily from the genera *Vibrio, Pseudomonas, Aeromonas* and *Alteromonas,* which appear to naturally reside within the oyster circulatory system (Olafsen et al., 1993; Garnier et al., 2007). This raises the questions of how hemocytes differentiate between pathogens and "natural" inhabitants and may be related to the function of pattern recognition receptor proteins (e.g. peptidoglycan recognition proteins) and antimicrobial peptides (AMPs) produced by these cells. Pattern recognition receptors are produced by oyster epithelial cells and hemocytes (Itoh and Takahashi, 2008) and when stimulated (by microbial products such as peptidoglycan) activate hemocytes, allowing them to migrate to the invasion site and express AMPs for a rapid and effective defence against invading microbes (Schmitt et al., 2012b). Additionally, the epithelial layer constitutively expresses a number of AMPs to further reduce microbial loads (Schmitt et al., 2012b).

Pathogens bypassing these initial defence strategies face phagocytosis by the circulating hemocytes in the hemolymph. Phagocytised pathogens (Canesi et al., 2002) are subsequently exposed to reactive oxygen species (ROS), enzymes and AMPs within the hemocyte (Labreuche et al., 2006a; Schmitt et al., 2012b). However, some bacterial and protozoan parasites are able to subvert intracellular degradation, effectively evading the oyster immune response (Schmitt et al., 2012c). This is primarily facilitated by the suppression of (ROS) generation, or reduced phagocytosis by the hemocytes (Schott et al., 2003; Labreuche et al., 2006b).

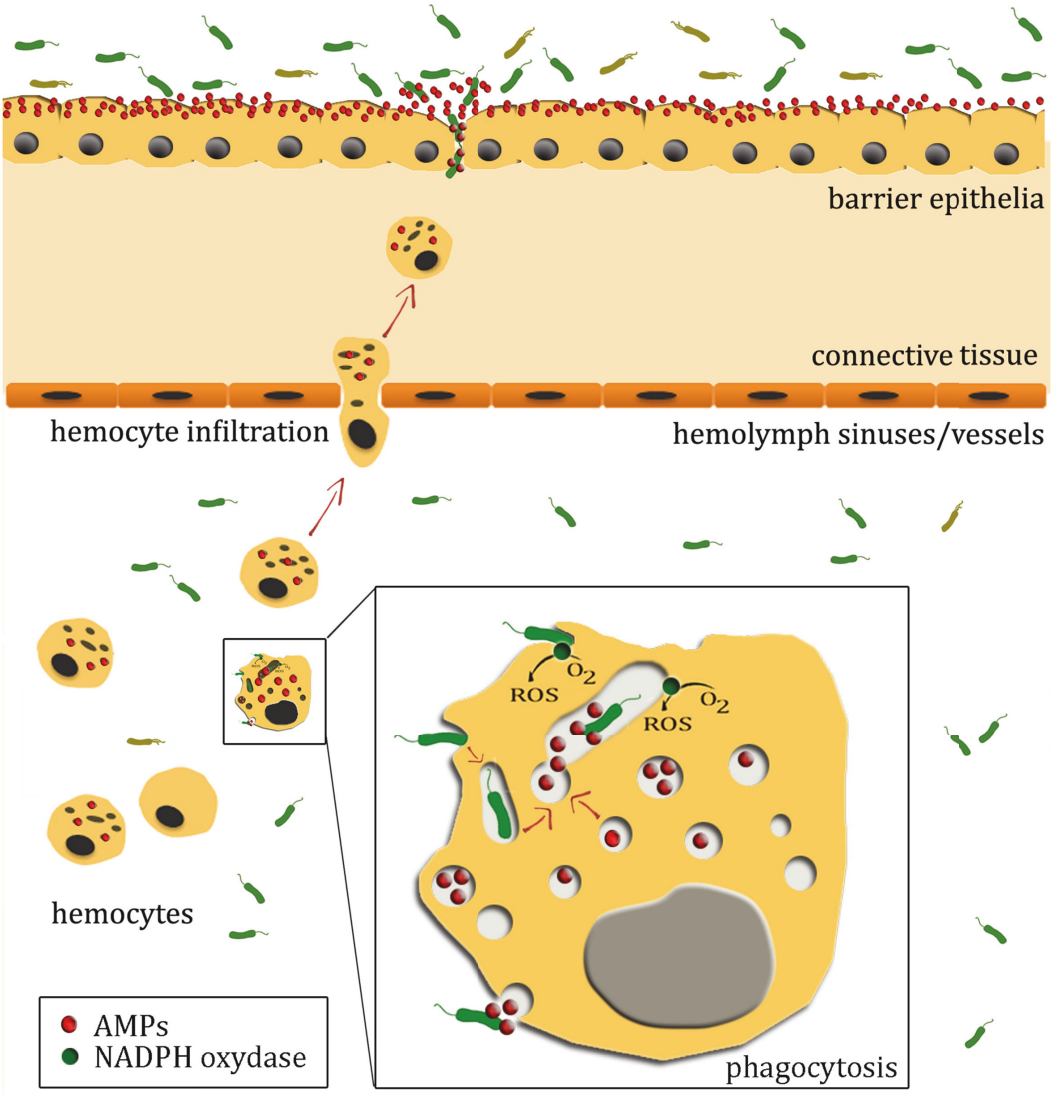


Figure 1.3: An overview of the oyster cellular immune response (Schmitt et al., 2012c) published by Frontiers in Microbiology. Invading pathogens must first bypass the epithelial layer, which produces antimicrobial peptides (AMP; red circle). Following this, the circulating hemocytes in the hemolymph engulf the microbial pathogens. They are then exposed to reactive oxygen species (ROS), which are produced by either NADPH oxidase (green circle) or the mitochondria, and antimicrobial proteins such as lysozyme and AMPs.

1.5 Diseases affecting oysters of economic importance

There are a number of well-characterised microbial diseases affecting several different oyster species. A summary of the known oyster diseases for each species is provided in Table 1.1.

Table 1.1: Diseases of economically important oyster species, their affected life stage and the pathology seen for each disease.

Oyster species	Disease/pathogen (agent)	Affected oyster stage	Pathology	Geographical distribution	Mortality range (%)	References
The Eastern oyster (*Crassostrea virginica*)	Dermo/*Perkinsus marinus* (Protozoan)	Adult	Tissue lysis, blockage of circulatory system	USA East Coast	20-85	(Andrews and Hewatt, 1957; Ford, 1996)
	MSX/*Haplosporidium nelsoni* (Protozoan)	Spat and adult	Epithelium infection, respiratory and digestive impacts	USA East Coast	33-95	(Haskin et al., 1966; Ford and Haskin, 1982; Ewart and Ford, 1993)
	ROD/*Roseovarius crassostreae* (Bacterium)	Spat	Mantle lesions, conchiolin deposits, tissue degradation	USA East Coast	54-75	(Bricelj et al., 1992; Boardman et al., 2008)
Sydney rock oyster (*Saccostrea glomerata*)	QX/*Marteilia sydneyi* (Protozoan)	Adult	Digestive tubule destruction, starvation	Australian East Coast	22-99	(Kleeman et al., 2002; Nell and Perkins, 2006)
	Winter Mortality/*Bonamia roughleyi* † (Protozoan)	Adult	Connective tissue disruption, ulcers, impaired muscle contractions, necrotic tissues	Australian East Coast	9-52	(Roughley, 1926; Mackin, 1959; Farley et al., 1988; Smith et al., 2000)
European flat oyster (*Ostrea edulis*)	Marteiliosis/*Marteilia refringens* (Protozoan)	‡	Digestive gland infection, impaired growth, starvation	France, Spain, Portugal and Greece	50-90	(Alderman, 1979; Virvilis and Angelidis, 2006; Bower, 2011; López-Sanmartín et al., 2015)
	Bonamiasis/*Bonamia ostreae* (Protozoan)	Adult, larvae	Gill and mantle lesions, parasite resides within hemocytes	France, Spain, England, Denmark, the Netherlands, USA West Coast	40-80	(Balouet et al., 1983; Elston, 1986)

Table 1.1 continued: Diseases of economically important oyster species, their affected life stage and the pathology seen for each disease.

Oyster species	Disease/pathogen (agent)	Affected oyster stage	Pathology	Geographical distribution	Mortality range (%)	References
Pacific Oyster (*Crassostrea gigas*)	Denman Island disease/ *Mikrocytos mackini* (Protozoan)	Adult	Green pustules, ulcers and abscesses on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	17-53	(Quayle, 1961; Farley et al., 1988; Elston et al., 2015)
	Nocardiosis/*Nocardia crassostreae* (Bacterium)	Adult	Green pustules and lesions on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	47-50	(Friedman et al., 1991)
	Vibriosis (Bacillary necrosis)/ *Vibrio* spp. (Bacterium)	Larvae, spat	Abnormal swimming, necrosis, lesions	Worldwide	76-100*	(Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002; Elston et al., 2008)
	Pacific Oyster Mortality Syndrome/OsHV-1 and OsHV-1 µ variant (Virus)	Larvae, spat	Lesions and cells with viral inclusions and hypertrophied nuclei. Reduced feeding and impaired swimming in larvae	USA East Coast, Australia, New Zealand, France, Sweden and Norway	40-100	(Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016)
	Summer Mortality/Unknown or multifactorial§	All stages	ill defined, characterised by high level mortalities during the warmer months	USA, France, Australia, Japan, Germany, Ireland, Sweden and Norway	30-100	(Mori, 1979; Soletchnik et al., 2005; Burge et al., 2007; Garnier et al., 2007; Malham et al., 2009)

†The aetiological agent of winter mortality may not be *Bonamia roughleyi*.
‡Age not reported, likely adult oysters are affected by marteiliosis as seen in QX disease.
§While no definite aetiological agent has been found, OsHV-1 and a number of *Vibrio* spp. have been associated with this disease usually during periods of host-stress (e.g. reproductive or heat stress).
*Depending on the *Vibrio* strain and bacterial concentration used.

1.5.1 Parasitic aetiological agents

Parasitic disease outbreaks have historically led to catastrophic losses of oysters, and large economic impacts. Dermo (also known as perkinsosis) and MSX are caused by the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* respectively (Mackin et al., 1950; Haskin et al., 1966). Specifically, historical outbreaks of dermo affecting *C. virginica* have contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). Both dermo and MSX are responsible for extensive annual mortality outbreaks, particularly along the east coast of America (Encomio et al., 2005). For *S. glomerata*, Queensland unknown disease (QX) is caused by the protozoan parasite, *Marteilia sydneyi* (Anderson et al., 1994; Kleeman et al., 2002), while the aetiolgical agent of *S. glomerata* winter mortality is unclear with conflicting morphological, histological and molecular evidence from different laboratories (Carnegie et al., 2014; Spiers et al., 2014). These two diseases have reduced cultivation in some Australian estuaries by as much as 97% (Nell and Perkins, 2006; O'Connor et al., 2008; Dove et al., 2013b). QX disease has been particularly harsh with mortality rates as high as 85-95% (Anderson et al., 1994; Bezemer et al., 2006). The decline of the *O. edulis* industry in Europe has been attributed to two parasitic diseases, marteiliosis (also known as Aber disease) and bonamiasis (Robert et al., 2013), caused by *Bonamia ostreae* and *Marteilia refringens* respectively (Alderman, 1979; Balouet et al., 1983; Elston, 1986).

1.5.1.1 Disease process of parasites

Parasitic diseases are chronic, typically taking weeks or months to kill their host through disruption of different tissue(s) usually causing effects such as oyster starvation, and/or tissue lysis (Andrews and Hewatt, 1957; Haskin et al., 1966; Balouet et al., 1983; Adlard and Ernst, 1995; Hervio et al., 1996).

This section will review what is known about parasitic infections of oysters including the oyster tissue(s) where infection is initiated, the process(es) by which parasites move to other tissues/sites in the oyster and, process(es) that lead to oyster death.

Of the various oyster parasites, the point/site of infection can vary and include the gill and palps for *M. sydneyi* (Kleeman et al., 2002) and, the mantle epithelium for *P. marinus* (Allam et al., 2013). However, for the remaining oyster parasites (*H. nelsoni*, *M. refringens*, *B. ostreae*, and *M. mackini*), the site(s) of infection are unknown and is an area that requires additional research. Despite this, gill infections are commonly observed for these parasites (Haskin et al., 1966; Balouet et al., 1983; Farley et al., 1988; Kleeman et al., 2002; Ragone Calvo et al., 2003; Carnegie and Burreson, 2011) indicating that oyster filter feeding is an important process for the transmission of the parasite into the oyster with the gills possibly acting as the point of infection.

Following initial infection, subsequent dissemination to specific tissues or cells varies depending on the infecting parasite, with hemocytes, the digestive gland and connective tissue known targets. *P. marinus* and *B. ostreae* are phagocytosed by the circulating hemocytes (Balouet et al., 1983; Schott et al., 2003) and are both able to survive the process through degradation or preventing the formation of toxic reactive oxygen species inside the hemocyte (Schott et al., 2003; Morga et al., 2009). These parasites are able to proliferate within the hemocyte and use them as a vehicle to spread throughout the oyster (Montes et al., 1994; Perkins, 1996) resulting in the lysis of various host tissues and/or blockage of the oyster circulatory system thus culminating in mortality (Andrews and Hewatt, 1957; Balouet et al., 1983; Choi et al., 1989; Encomio et al., 2005). For the two *Marteilia*

parasites, *M. sydneyi* and *M. refringens,* both lead to an infection of the digestive gland resulting in disrupted growth and impaired nutrient uptake leading to oyster starvation and mortality (Alderman, 1979; Camacho et al., 1997; Kleeman et al., 2002; Green et al., 2011). Destruction of the digestive gland and tubules is also observed for oysters infected with *H. nelsoni* (Ford and Haskin, 1982), but it is not clear whether the parasite also affects nutrient uptake similar to the *Marteilia* parasites. While it is known that systemic dissemination of *M. sydneyi* cells follows on from the initial gill and palp infection (Kleeman et al., 2002), it is unclear whether *M. refringens* and *H. nelsoni* also disseminate towards the digestive gland/tubules from an initial infection site, or whether the infection is initiated in the digestive gland/tubules. Connective tissue cells (cells between organ tissues) of the oyster are infected by *M. mackini* causing mortality through tissue disruption and necrosis (Hervio et al., 1996; Bower et al., 1997). This process produces characteristic green pustules, ulcers and abscesses on several different oyster tissues (Figure 1.4) (Farley et al., 1988; Hervio et al., 1996).

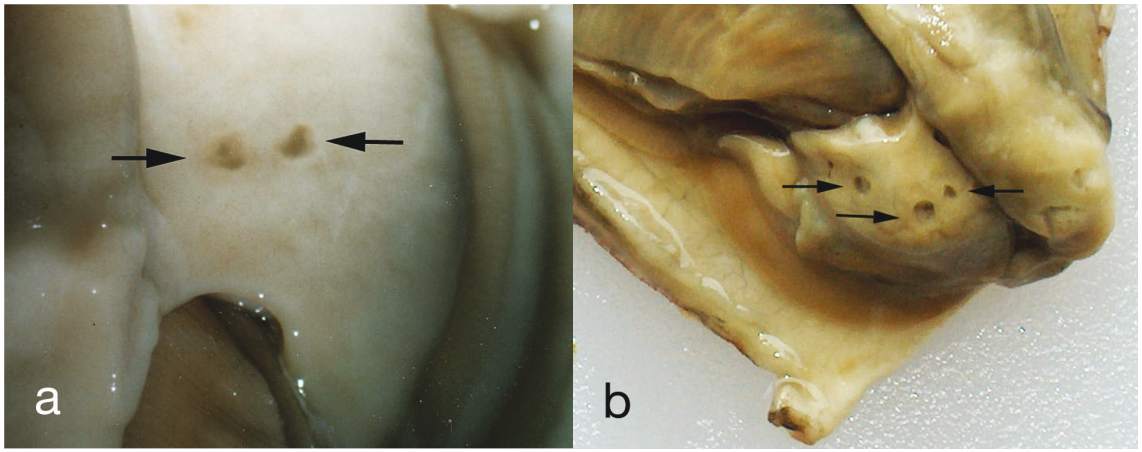


Figure 1.4: Ulcerated lesions (black arrows) on the labial palps of *Crassostrea gigas* characteristic of Denman Island Disease (Elston et al., 2015), published by Diseases of Aquatic Organisms, © Inter-Research 2015.

Since the aetiological agent(s) of winter mortality is still being debated (Spiers et al., 2014), the disease process remains poorly understood. Spiers et.al. (2014) carried out a longitudinal study with the aim of determining the aetiological agent of winter mortality. While the presence of a *Bonamia* spp. was confirmed by PCR, the occurrence of this parasitic organism was quite low (3% of all samples) and the 18S rRNA sequence of the observed protozoan was closely related to another organism, *B. exitiosa*, which has previously been identified in *S. glomerata* (Carnegie et al., 2014) but not in association with clinical disease. The low prevalence of *Bonamia* spp. DNA in the Spiers et al. study was inconsistent with the high prevalence of pathological observations. Similarly, no *Bonamia* spp. was found within the lesions of the oysters (Spiers et al., 2014). While this research suggests that another organism may be causing or perhaps working with *Bonamia* spp. in winter mortality, this study only observed a 10% total mortality over the entire study period, which is not an extensive outbreak. As a result, further studies are required to elucidate the aetiological agent(s) of winter mortality before further research on the disease process can be elucidated.

1.5.1.2 Environmental reservoirs and transmission of infectious parasites

For the majority of infectious parasites, the environmental reservoir and details of transmission to and between oysters is not completely understood. On reservoirs, it is unknown whether the parasite is residing in the environment (i.e. the water column or in sediments), or whether an intermediate host is acting as an environmental reservoir. It may also be possible that the parasite is using the intermediate host for maturation and then residing in another unknown organism. For example, *M. sydneyi* spores are only able to survive in the marine environment for up to 35 days, which

is inconsistent with the yearly cycle of QX disease outbreaks (Wesche et al., 1999). It is therefore likely that an intermediate host exists as a reservoir of the parasite. Recent evidence suggests that *M. sydneyi* is present within the intestinal epithelium of the marine worm *Nephtys australiensis* and it has been proposed that this organism may act as a reservoir for *M. sydneyi* or may be critical for the maturation and transmission of *M. sydneyi* (Adlard and Nolan, 2015). Therefore, further research is necessary to determine where these parasites reside, and for those parasites with intermediate hosts, whether their intermediate host may act as that reservoir.

In regards to transmission, parasites can either be transmitted directly or via an intermediate. Direct transmission of parasites between infected and naïve oysters has been observed for dermo, bonamiasis, and Denman island disease (Elston, 1986; Quayle, 1988; Ewart and Ford, 1993; Hervio et al., 1996). While the causative agents of MSX, QX, and marteiliosis require an intermediate host(s) for the maturation and transmission of the parasite.

For those directly transmitted parasites, *P. marinus* is shed into the water column from infected oyster hosts, which can then be ingested by neighbouring oysters (Ewart and Ford, 1993). Similarly, only cohabitation with infected oysters is necessary for the transfer of *B. ostreae* and *M. mackini* to naïve hosts (Elston, 1986; Quayle, 1988; Hervio et al., 1996). The larvae of *O. edulis* can also be infected with *B. ostreae*, potentially allowing them to act as a reservoir of the parasite in the environment (Arzul et al., 2011).

For those parasites with no direct transmission, early laboratory-based studies were unsuccessful in transmitting *H. nelsoni* to uninfected oysters through co-incubation with infected oysters (Ewart and Ford, 1993). Later

studies have demonstrated that an intermediate carrier capable of penetrating 1 mm^2 filters is required for transmission to naïve oysters (Sunila et al., 2000). Similarly, while field studies investigating the transmission of *M. refringens* into *O. edulis* demonstrated that the parasite was transmissible through cohabitation of uninfected with infected oysters or by deploying uninfected oysters in areas known to contain the pathogen (Berthe et al., 1998), laboratory-based cohabitation experiments and inoculations were insufficient to cause infections (Berthe et al., 1998). Later studies have identified two copepod species, *Paracartia grani* and *Paracartia latisetosa,* harbouring *M. refringens* and are implicated in the transmission of this parasite (Audemard et al., 2002; Arzul et al., 2014). This is similar for *M. sydneyi,* in which before an infected oyster dies, almost all of the *M. sydneyi* sporonts (Figure 1.5) are shed into the environment (Roubal et al., 1989). However, direct transmission studies have been unable to transmit the parasite to naïve oysters (Lester, 1986). Likely the intermediate host, *Nephtys australiensis*, and possibly other unknown hosts, are needed to transmit *M. sydneyi* to naïve oysters (Adlard and Nolan, 2015).

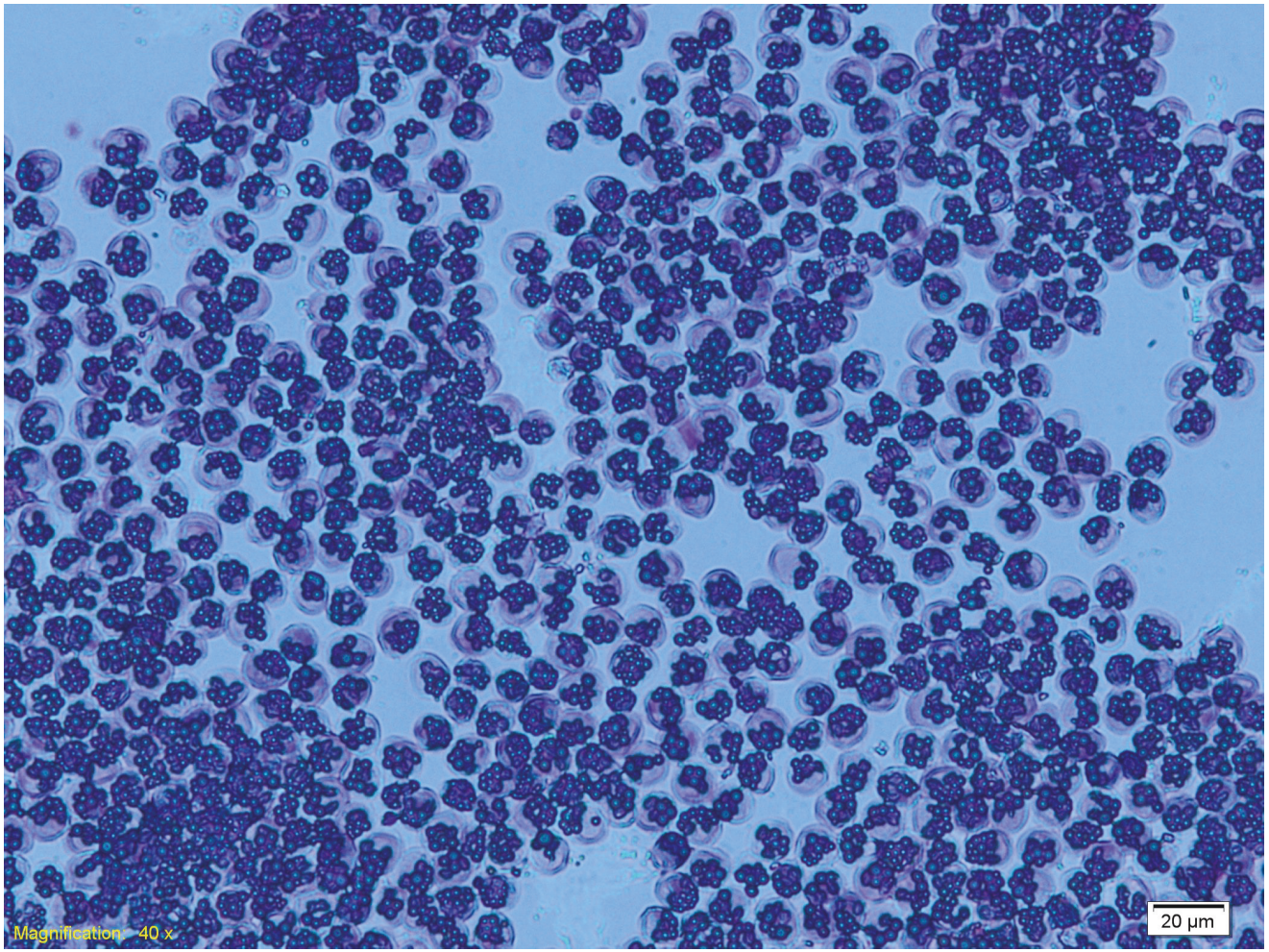


Figure 1.5: Purified *Marteilia sydneyi* sporonts, the causative agent of QX disease of *Saccrostrea glomerata*. Image is at 40x magnification. Image produced by Cheryl Jenkins and Jeffrey Go at the New South Wales Department of Primary Industries.

1.5.1.3 Management strategies of parasitic diseases

Attempts to reduce the impact of these parasitic diseases revolve around the development of breeding programs, modified husbandry practices, and quarantining affected areas (Nell et al., 2000; Smith et al., 2000; Ragone Calvo et al., 2003; Green et al., 2011; Lynch et al., 2014). Of these strategies, breeding for disease-resistance has been the most successful (Ragone Calvo et al., 2003; Dove et al., 2013a; Dove et al., 2013b; Lynch et al., 2014). Dual resistance has been bred into *C. virginica* against dermo and MSX disease, leading to an improved survivability of approximately 30-60% when compared to control oyster stocks (Ragone Calvo et al., 2003). Similarly, a breeding programme carried out in Ireland since 1988 has successfully mitigated the damage of *B. ostreae* on *O. edulis* populations, culminating in an increased survival rate of 75% of market sized adult oysters, relative to 5-10% before the breeding programme began (Lynch et al., 2014). Breeding for disease-resistance has also been successful for *S. glomerata* against QX and winter mortality, with oyster mortality decreasing from 97% to 28% for QX, and 52% to 23% for winter mortality (Dove et al., 2013b). Modified husbandry practices are used to limit the exposure time of the oyster to the parasite, this can be done by altering the growing height of the oysters, or by transplanting new oysters after the disease period has passed. Modified husbandry practices can be seen with winter mortality, in which *S. glomerata* are grown at a position located 15-30 cm higher in the tidal range than the typical growth height (approximately mid-tide level) (Smith et al., 2000).

1.5.2 Bacterial aetiological agents

1.5.2.1 Disease process of bacterial pathogens

Bacterial disease outbreaks are often sudden, resulting in severe mortality in a matter of days or weeks (Jeffries, 1982; Friedman and Hedrick, 1991; Bricelj et al., 1992). *Roseovarius crassostreae*, the aetiological agent of ROD in *C. virginica* causes sporadic outbreaks during the summer months, with mortalities up to 75% seen (Bricelj et al., 1992). For vibriosis of *C. gigas*, mortalities can exceed 90% within a period of only 24 hours (Takahashi et al., 2000). While *Nocardia crassostreae* the causative agent of *C. gigas* acts slower, resulting in mortalities up to 47% over 34 days (Friedman and Hedrick, 1991).

Lesions are common symptoms for oysters affected by ROD, nocardiosis, and vibriosis, and spat are often the most at risk for infection (Jeffries, 1982; Bricelj et al., 1992; Bower, 2006). In addition, *R. crassostreae* colonises the inner shell surface of *C. virginica*; the oyster responds to this intrusion through the formation of conchiolin (organic compound secretions involved in shell formation) deposits on the shell, which is thought to act as a barrier to contain further bacterial infection (Boardman et al., 2008). Additional pathological symptoms include lesions on the mantle, degradation of muscles and tissues, infiltration of hemocytes into the epithelium of the oyster, as well as lesions under the hinge ligament (Bricelj et al., 1992). Conchiolin deposits filled with bacteria and necrotic cells are also observed in vibriosis of *C. gigas* (Ralph et al., 1999). Conversely, conchiolin deposits aren't seen in nocardiosis, instead oysters display green pustules and lesions on a number of different oyster tissues (Bower, 2006).

A number of different *Vibrio* species cause disease in *C. gigas*, resulting in either vibriosis or bacillary necrosis (Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002). A summary of the known *Vibrio* pathogens can be seen in Table 1.2. *C. gigas* larvae and spat are typically affected by *Vibrio* infections (Jeffries, 1982; Elston et al., 2008). Vibriosis in oyster larvae involves tissue necrosis (Figure 1.6) and abnormal swimming culminating in mortality (Jeffries, 1982). Vibriosis of spat can lead to lesions and necrosis of the tissues (Elston et al., 2008). As seawater temperatures rise with climate change, the spread and growth of bacteria such as *Vibrio,* which prefer warmer waters, has been predicted to be enhanced (Martinez-Urtaza et al., 2010; Vezzulli et al., 2016). Notably, an elevation in surface seawater temperature was linked to the resurgence of the oyster pathogen *Vibrio coralliilyticus* on the North American Pacific Coast, where it was responsible for a major *C. gigas* mortality event (Elston et al., 2008; Richards et al., 2015).

Table 1.2: *Vibrio* pathogens of *Crassostrea gigas* and their affected life stage. Bacterial pathogens are typically isolated from diseased oysters and used in virulence assays to determine pathogenicity.

Bacterial agent	Stage affected	Reference
V. tubiashii	Larvae	(Jeffries, 1982; Hada et al., 1984; Takahashi et al., 2000)
V. splendidus	Larvae	(Sugumar et al., 1998)
	Spat	(Waechter et al., 2002)
	Adult	(Garnier et al., 2007)
V. alginolyticus	Larvae	(Luna-González et al., 2002)
	Adult‡	(Go et al., 2017)
V. splendidus group	Spat	(Gay et al., 2004)
	Adult	(Garnier et al., 2007)
V. aestuarianus	Spat	(Saulnier et al., 2009; Saulnier et al., 2010)
	Adult	(Garnier et al., 2007; Saulnier et al., 2010)
V. lentus	Spat	(Saulnier et al., 2010)
V. harveyi	Spat	(Saulnier et al., 2010)
	Adult‡	(Go et al., 2017)
V. coralliilyticus	Spat	(Elston et al., 2008; Richards et al., 2015)
V. crassostreae	Spat†	(Lemire et al., 2015; Bruto et al., 2017)
	Adult‡	(Go et al., 2017)

†Based on supplementary information for the production of specific pathogen free (SPF) oysters

‡Used in an inoculation cocktail comprised of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and *V. crassostreae*

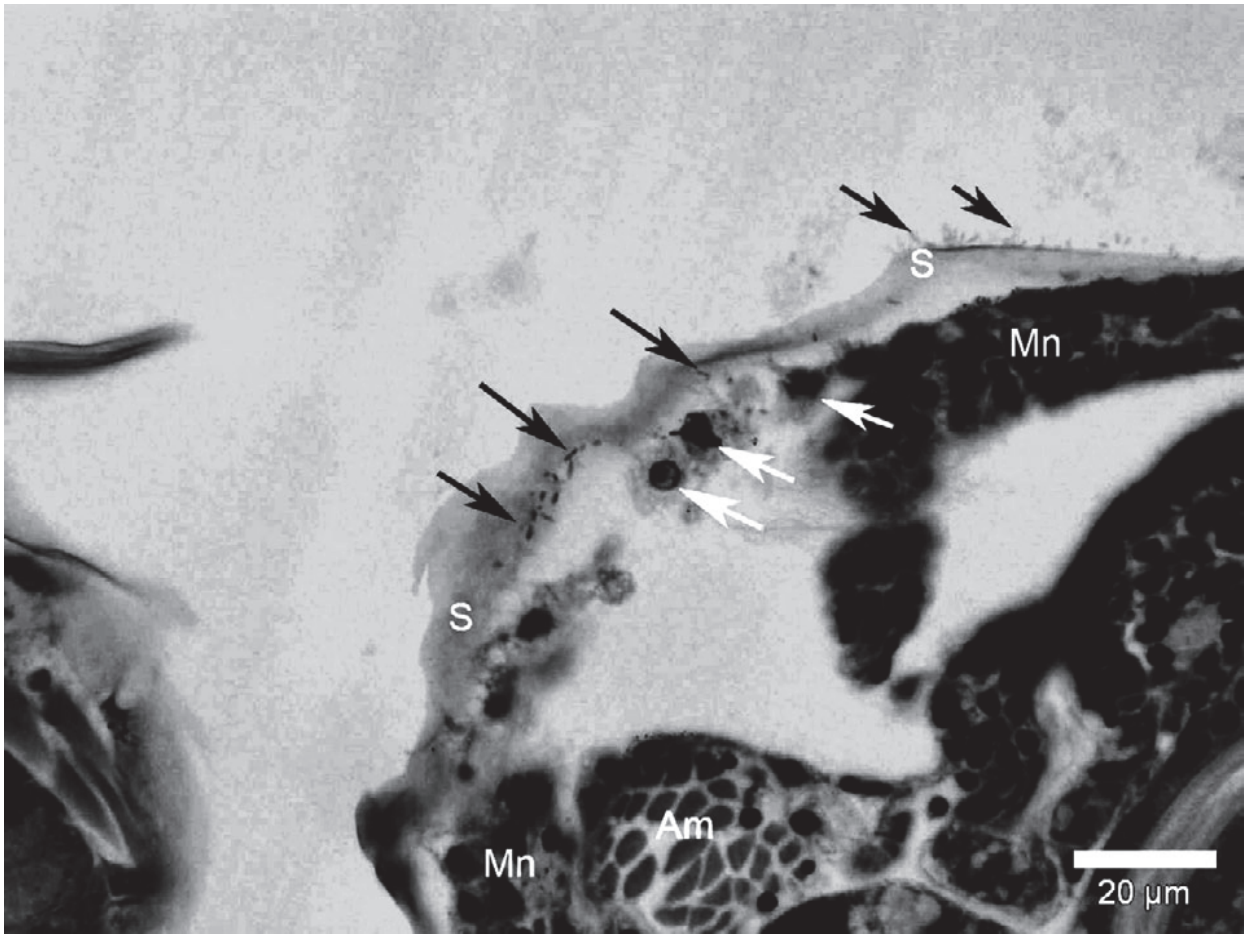


Figure 1.6: Histological section of *Crassostrea gigas* larvae with a persistent *Vibrio* infection (black arrows), as well as necrotic epithelial cells (white arrows). Larvae tissue are marked as S (shell), Mn (mantle) and Am (adductor muscle) (Elston et al., 2008). Published by Diseases of Aquatic Organisms, © Inter-Research 2008.

While vibriosis tends to affect larvae and spat, experimental injections of adult oysters with *Vibrio* species, including *V. aestuarianus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Garnier et al., 2007; Saulnier et al., 2010; Go et al., 2017) has also been shown to induce mortality, with a weakening of the adductor muscle and necrotic oyster tissues observed (Garnier et al., 2007). However, the injection of bacteria into oyster hemolymph/tissues may not be a good model for the natural transmission of *Vibrio* infections in the environment. Often *Vibrio* infections, particularly from the *V. splendidus* group, are found to occur concurrently with a herpesvirus infection (OsHV-1) (Segarra et al., 2010; Pernet et al., 2012; Keeling et al., 2014; De Lorgeril et al., 2018) with a recent study highlighting a synergistic, polymicrobial infection process, in which the oyster immune system is suppressed following OsHV-1 infection, allowing for bacteraemia to occur (De Lorgeril et al., 2018).

1.5.2.2 Environmental reservoirs and transmission of bacterial pathogens

Often, bacterial infections are opportunistic, requiring an environmental stressor or immune suppression of the oyster host before infection occurs (Bricelj et al., 1992; De Lorgeril et al., 2018). No studies have identified environmental reservoirs for *N. crassostreae* and *R. crassostreae*, while *Vibrio* species are ubiquitous in the environment and are commonly found in the water column, sediments, vegetation, and associated with other organisms (Vezzulli et al., 2010; Chase et al., 2015). Given the worldwide distribution of vibriosis, it is possible that *Vibrio* bacteria are members of the oyster microbiome that are awaiting favourable conditions to cause disease, such as with OsHV-1 infection (De Lorgeril et al., 2018) or with the acquisition of virulence plasmids through horizontal gene transfer (Bruto et

al., 2017). Whereas *N. crassostreae* and *R. crassostreae* are localised to the USA northwest coast and USA east coast respectively (Friedman et al., 1991; Bricelj et al., 1992), because of this, there likely exists an unknown seasonal environmental reservoir for these pathogens.

Regarding transmission, laboratory transmission studies of ROD indicate that *R. crassostreae* is transmissible with symptoms arising 3 to 7 weeks after cohabitation with infected oysters (Lewis et al., 1996). Conversely, laboratory transmission of *N. crassostreae*, has not been successful (Friedman et al., 1991) suggesting either an unknown transmission element is required to infect new oysters, or that the infection is opportunistic, requiring environmental stressors such as the high temperatures typically seen during summer months, in order to induce disease (Friedman et al., 1991). Transmission of *Vibrio* species from infected to naïve oysters is likely bacterial species dependent. While one study was able to cause vibriosis in naïve animals by cohabiting them with oysters injected with a mixture of *V. splendidus* and *V. aestuarianus* (De Decker and Saulnier, 2011), another study was unable to transmit vibriosis when using a *Vibrio* cocktail made of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Go et al., 2017) possibly contrasting a difference in experimental methodology, or a difference between the transmission of different *Vibrio* species.

1.5.2.3 Management strategies for bacterial pathogens

No control measures are currently employed to contain nocardiosis of *C. gigas* or for ROD of *C. virginica*. Often vibrio blooms due to favourable environmental conditions (warm water and excess nutrients) are the cause of vibriosis for larvae and spat in hatchery settings (Elston et al., 2008). Monitoring environmental conditions and water quality may help predict

Vibrio outbreaks, possibly allowing farmers to change their water source in hatchery settings, or to remove oysters from the environment until the bloom has passed.

1.5.3 Viral aetiological agents

Of these economically valuable oyster species, only one virus, ostreid herpesvirus 1 (OsHV-1), has been identified as a major disease-causing pathogen (Hine et al., 1992; Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). OsHV-1 primarily infects and induces mortality in *C. gigas* larvae and spat, as well as young adult oysters, with observed mortality rates ranging between 40 to 100% (Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010). OsHV-1 has been linked to a number of large mortality events across the globe and is continuing to spread (Burge et al., 2006; Segarra et al., 2010; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). Oysters infected with OsHV-1 display both lesions and cellular infections throughout the gills, mantle, digestive glands and in the hemocytes, whereby cells show altered cellular morphology, such as abnormal shapes, enlarged nuclei, nuclear fragmentation and nuclear inclusions (Hine et al., 1992; Renault et al., 1994; Friedman et al., 2005). OsHV-1 infected larvae have also been observed to have reduced feeding capacity and impaired swimming abilities (Hine et al., 1992; Renault et al., 2001).

Since its characterisation, a number of variant forms of OsHV-1 have been discovered (Arzul et al., 2001; Segarra et al., 2010; Martenot et al., 2011). Of these, a micro-variant form, named OsHV-1 μvar (Segarra et al., 2010), has been associated with mortality outbreaks in a number of countries

(Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016). This micro-variant form has a number of nucleotide substitutions and deletions that distinguish it from the original variant (Segarra et al., 2010). Infection by OsHV-1 μvar acts to suppress the oyster's immune system thereby allowing opportunistic bacteria (such as *Vibrio* bacteria) to cause bacteraemia (De Lorgeril et al., 2018), and the oyster microbiome also shifts in response to viral infection (De Lorgeril et al., 2018). Furthermore, treating OsHV-1 μvar infected oysters with antibiotics significantly reduces the number of mortalities (Petton et al., 2015). As the oyster microbiome can act as a source of opportunistic pathogens (Lokmer and Wegner, 2015), further studies are required to examine the relationship (and possible interactions) between OsHV-1 μvar and the oyster microbiome.

OsHV-1 has been experimentally transferred to naïve oysters within the laboratory (Dégremont et al., 2013; Petton et al., 2015). Notably, it has also been demonstrated that OsHV-1 resistant oysters infected with OsHV-1 are unable to transmit the virus to naïve oysters, and resistant oysters maintained an overall lower viral load than non-resistant oysters (Dégremont et al., 2013). Management strategies have been focused on movement controls (quarantining affected areas) and the production of genetic lines of oysters resistant to OsHV-1, that are able to reduce viral replication and more easily recover from viral infection (Segarra et al., 2014).

1.5.4 Unknown aetiological agents

In recent decades a phenomenon known as 'summer mortality' has heavily impacted the *C. gigas* aquaculture industry globally. These disease outbreaks have occurred all over the world including France (Garnier et al., 2007; Segarra et al., 2010), Australia (Jenkins et al., 2013; Go et al., 2017), the

USA (Friedman et al., 2005), Germany (Watermann et al., 2008), Ireland (Malham et al., 2009), Japan (Mori, 1979) and in recent years Sweden and Norway (Mortensen et al., 2016). Summer mortality is marked by the loss of over 30% of oyster stocks (Soletchnik et al., 2005; Soletchnik et al., 2007) and in some instances has been observed to result in 100% mortality (Burge et al., 2007). Summer mortality has been responsible for catastrophic losses of *C. gigas* harvests since the 1960's (Mori, 1979), but the mechanisms involved and if a pathogen(s) is responsible remains largely unknown. A number of different factors have been implicated in these mortalities, including rising seawater temperatures, eutrophication, infections by *Vibrio* species and the herpesvirus OsHV-1. However, the cause appears to be multifactorial (Malham et al., 2009; Dégremont et al., 2013; Lemire et al., 2015; Petton et al., 2015) involving the interplay of multiple biotic and abiotic factors, which may affect the oyster immune system allowing opportunistic pathogens to take hold (Samain et al., 2007; Malham et al., 2009), and/or the abundance and virulence of pathogens. In this sense, summer mortality is an umbrella term that likely encompasses a number of different diseases with known or unknown aetiological agents. The bulk of recent research suggests a major role for OsHV-1 in summer mortality, with many research groups detecting this virus when disease outbreaks occur (Friedman et al., 2005; Burge et al., 2006; Burge et al., 2007; Segarra et al., 2010; Jenkins et al., 2013). It is notable however, that OsHV-1 was not detected in a recent summer mortality event in Australia (Go et al., 2017). Likely, periods of high temperature and low salinity acted to stress the oyster, resulting in immune suppression (Go et al., 2017), and allowing for bacterial infection to occur. This is evidenced with OsHV-1, in which infection acts to suppress the oyster's immune system allowing for bacteraemia to kill the host (De Lorgeril et al., 2018).

1.6 The role of the environment in facilitating disease outbreaks

The environment within which an organism resides, the pathogens to which it is exposed to, and the host's physiology (including the microbiome) can be considered an "interactome" that influences disease dynamics (Figure 1.7) (Arthur et al., 2017). The concept of the interactome is particularly relevant to oysters given that they filter large quantities of water, thereby increasing the chance of exposure to pathogens. However, while there has been a substantial amount of research into the mechanisms behind diseases of oysters due to the global economic importance of these species, only recently have studies taken a more holistic approach to unravelling the interactome (Pernet et al., 2016). As a result, there is a need to move beyond viewing oyster diseases from a classical perspective (Koch's postulates; one disease one pathogen), to a more ecological viewpoint of disease.

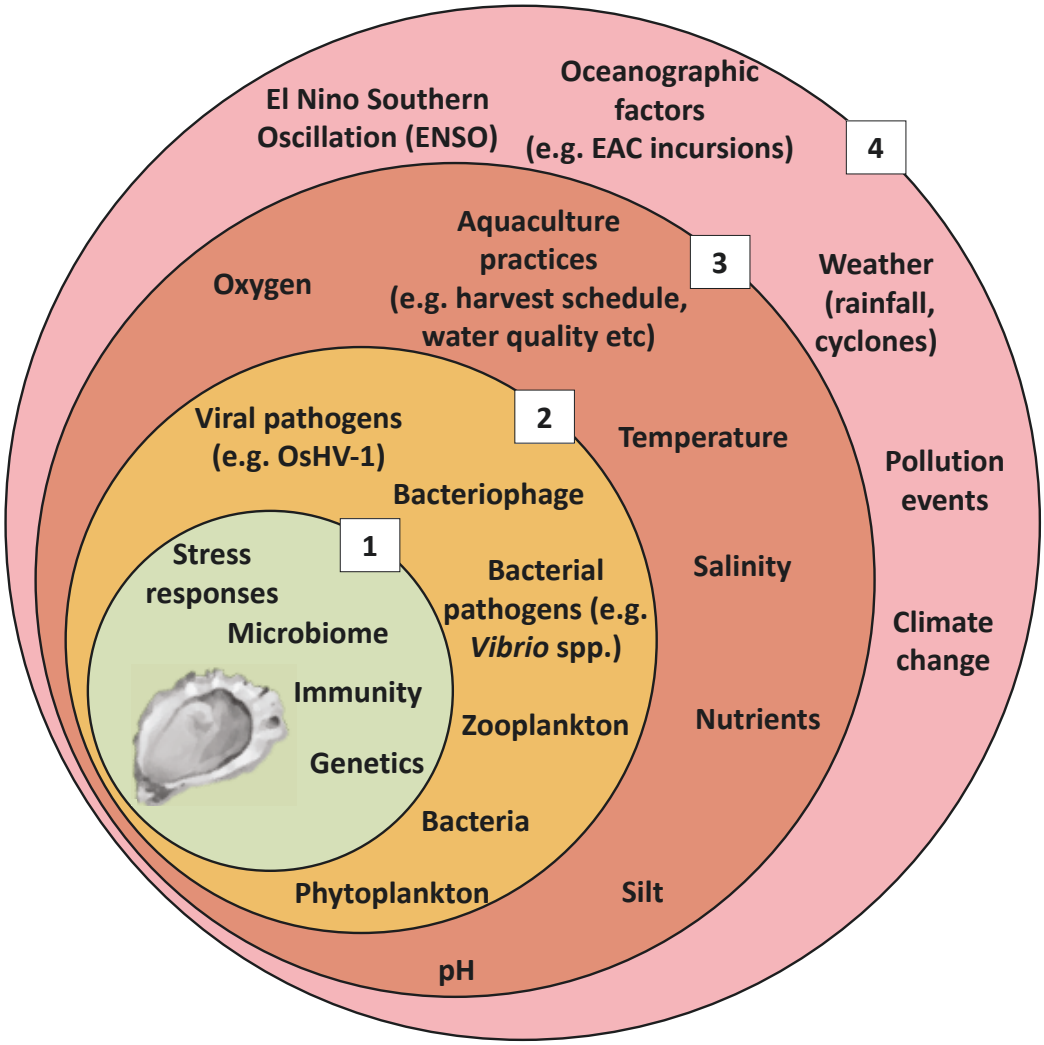


Figure 1.7: The interactome/synergism of oyster diseases. The outer rings are large scale environmental events (e.g. climate change) that influence the lower rings (e.g. temperature) allowing for a cascade effect that eventually influences microbial communities and pathogens (e.g. increased pathogen proliferation), that can then act on the oyster host.

There is growing evidence that environmental factors are critical in the spread and severity of oyster diseases (Ford, 1996; Petton et al., 2013; Mortensen et al., 2016). A summary of the environmental parameters that have been found to influence oyster diseases is presented in Table 1.3.

Table 1.3: Environmental factors that influence oyster diseases

Disease	Influential environmental parameters	References
Dermo	Increased winter temperature Increased salinity	(Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Soniat et al., 2012)
MSX	Increased winter temperature Increased salinity	(Haskin and Ford, 1982; Ford et al., 1999)
ROD	Increased temperature Increased salinity	(Lewis et al., 1996)
QX	Increased temperature Decreased salinity for spores	(Wesche et al., 1999)
Winter mortality	Dry autumns Increased salinity Decreased temperature	(Roughley, 1926; Butt et al., 2006; Nell and Perkins, 2006)
Marteiliosis	Increased temperature	(Berthe et al., 1998; Audemard et al., 2001)
Bonamiasis	Decreased temperature Increased salinity Higher pH‡	(Arzul et al., 2009)
Denman Island disease	Decreased temperature	(Hervio et al., 1996; Bower et al., 1997)
Nocardiosis	Increased temperature Lower dissolved oxygen	(Friedman et al., 1991; Engelsma et al., 2008)
Vibriosis	Higher temperature to increase *Vibrio* growth Low salinity inhibits *Vibrio* infectivity	(Lacoste et al., 2001; Elston et al., 2008; Richards et al., 2015)
OsHV-1	Increased temperature for viral replication Increased temperature for viral transmission Rainfall	(Jenkins et al., 2013; Petton et al., 2013; Renault et al., 2014)
Summer mortality	Chlorophyll *a* Temperature Turbidity Salinity Nutrients (Ammonium, Phosphate, Nitrate, Nitrite, Silicate)	(Soletchnik et al., 2007; Malham et al., 2009)

‡Observation made by the authors that more acidic media increased parasite mortalities.

1.6.1 Temperature

In marine environments, sea temperature is a major driver of oyster disease outbreaks with temperature shifts mostly dictated by the seasons, although oceanic phenomena (such as marine heat waves) can also play a role (Table 1.3). Warmer temperatures are known to affect the severity and prevalence of dermo, MSX, ROD, marteiliosis, QX, nocardiosis, vibriosis, OsHV-1 and summer mortality, while bonamiasis is most prominent during cooler water temperatures (Ford, 1996; Lewis et al., 1996; Wesche et al., 1999; Arzul et al., 2009; Malham et al., 2009; Green et al., 2011; Petton et al., 2013). As a result, marteiliosis, nocardiosis, summer mortality (including OsHV-1), MSX and ROD disease outbreaks occur, or are more severe, during the summer months (Friedman et al., 1991; Berthe et al., 1998; Boettcher et al., 1999; Friedman et al., 2005; Soletchnik et al., 2007; Engelsma et al., 2008; Watermann et al., 2008), with outbreaks of vibriosis occurring during unusually warmer than normal summer temperatures (Lacoste et al., 2001; Elston et al., 2008). Where cooler temperatures would normally supress disease, there is evidence that unusually warm winters are a catalyst for increased intensity of dermo and MSX outbreaks in the following summer (Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Ford et al., 1999). It's not always clear why warmer temperatures induce disease outbreaks, but there is evidence that enhanced pathogen replication, transmission, and stress to the host are likely determinants (Taylor, 1983; Gilad et al., 2003; Lokmer and Wegner, 2015; Tout et al., 2015).

Laboratory- and field-based studies have identified clear temperature thresholds that facilitate pathogen transmission. For the pathogens *R. crassostreae, M. refringens* and OsHV-1, the highest levels of transmission occur at temperatures greater than 18 °C (Lewis et al., 1996), 17 °C,

(Audemard et al., 2001) and 13.4 °C (Petton et al., 2013) respectively. In the field, disease outbreaks by these pathogens occur at slightly elevated temperatures, exceeding 20 °C for ROD and marteiliosis (Berthe et al., 1998; Boettcher et al., 1999), and 16 °C for OsHV-1 (Renault et al., 2014), indicating that pathogen colonisation is only one aspect of disease causation and that conditions that favour growth and increased host susceptibility also drive outbreaks. Consistent with this, ROD disease onset is reduced from 7 weeks at the permissible temperature of 18 °C to only 3 weeks when the temperature is increased to 25.9 °C, following transmission at 18 °C (Lewis et al., 1996). Regarding effects on the oyster host, warmer temperatures of 21 °C are sufficient to reduce the numbers of hemocytes in the *C. gigas* hemolymph, as well as reducing their phagocytic ability, as was demonstrated by oyster hemocytes challenged with *V. anguillarum* (Malham et al., 2009).

In contrast to the examples above, some pathogens have greater impacts under cooler temperatures. The viability of *M. sydneyi* spores is highest when temperature is reduced from 25 °C to 15 °C (Wesche et al., 1999), while *B. ostreae* shows improved survivability at 4 °C compared to temperatures at 15 °C and above (Arzul et al., 2009). Furthermore, outbreaks of winter mortality disease routinely occur in late winter or early spring (Roughley, 1926; Spiers et al., 2014).

1.6.2 Salinity

Salinity shifts have been implicated as key factors in outbreaks of dermo, MSX, ROD, QX, bonamiasis, vibriosis and summer mortality. Each oyster species has an optimal salinity concentration for growth, with 15-18 ppt (parts per thousand), 20-25 ppt, 20 ppt and 25-35 ppt being the optimal range

for *C. virginica*, *C. gigas*, *O. edulis* and *S. glomerata* respectively (Nell and Holliday, 1988; Wallace, 2001; FAO, 2016a; c). Shifts from these optimal ranges can occur following rainfall events, periods of extended drought, tidal changes and from wind-driven flow (Geyer, 1997; Drexler and Ewel, 2001; Schmidt and Luther, 2002; Da Costa et al., 2016). Infections from Dermo routinely occur at salinities above 9 ppt, with the greatest infections occurring above 15 ppt (Burreson and Ragone Calvo, 1996), which is within the optimal range of growth for *C. virginica* (Wallace, 2001), although once an oyster is infected, the infection can persist under salinity levels as low as 1-13 ppt (Andrews and Hewatt, 1957). Long periods of minimal rainfall also lead to an increase in dermo disease intensity and prevalence, which is thought to be related to increased salinity levels (Soniat et al., 2012).

For *P. marinus* (>15 ppt) and *H. nelsoni* (>15 ppt), infections occur within the optimal range of growth for their host (15-18 ppt for *C. virginica*). MSX disease severity is increased when the salinity is greater than 15 ppt, which is also within the optimal salinity range for *C. virginica* (Haskin and Ford, 1982). The protozoan, *B. ostreae* and the spores of *M. sydneyi* prefer high salinity (Wesche et al., 1999; Arzul et al., 2009). *M. sydneyi* spores showing heightened viability with increasing salinity, with an optimum viability at 34 ppt (Wesche et al., 1999) corresponding to the optimal salinity range of 25-35 ppt for *S. glomerata*. *B. ostreae* shows greatest survival in salinities greater than 35 ppt (Arzul et al., 2009), which is beyond the optimal salinity concentration (20 ppt) for *O. edulis*.

Salinity levels can also impact bacterial diseases such as ROD and vibriosis. Transmission of ROD readily occurs at salinities greater than 18 ppt, the upper limit for *C. virginica*, and while infections do occur at lower salinities (10 ppt and 14 ppt) mortality rates are significantly decreased (Lewis et al.,

1996). Conversely, mortality from *V. coralliilyticus* and *V. tubiashii* infection in *C. virginica* decreased from 100% and 70.7% respectively to 0% by reducing the salinity levels from 28 ppt to 9.6 ppt (Richards et al., 2015). Rates of summer mortality are also correlated with low salinity, with oyster mortalities the greatest during the low autumn-winter salinity period (Soletchnik et al., 2007).

With the exception of *B. ostreae*, the salinity concentrations that allow for infections by the protozoans are within the optimal range for their host. While bacterial infection and mortality caused by *R. crassostreae* (>18 ppt), *V. coralliilyticus* (28 ppt) and *V. tubiashii* (28 ppt) all occur outside the hosts optimal salinity range (15-18 ppt) possibly indicating that bacteria require an external stressor to allow for disease progression to occur, while protozoan parasites do not.

1.6.3 Dissolved oxygen and pH

N. crassostreae induced mortalities are correlated with lower dissolved oxygen concentrations, possibly through an impact on the hosts ability to combat this pathogen (Engelsma et al., 2008). In addition, hypoxic environments have been shown to increase the acquisition and infection intensity of *P. marinus* infections in *C. virginica* (Breitburg et al., 2015; Keppel et al., 2015), while pH does not appear to play a role in *P. marinus* infection dynamics (Keppel et al., 2015). Decreased pH levels also significantly affect the formation and dissolution of the *C. virginica* shell, which can potentially increase oyster susceptibility to disease and predation (Waldbusser et al., 2011a; Waldbusser et al., 2011b). The combination of decreased pH and a hypoxic environment reduces the ability of hemocytes to create reactive oxygen species (Boyd and Burnett, 1999), which would

ultimately hamper their ability to combat microbial infections. Previous studies have shown that acidification of water (<pH 5.5) from acid sulphate soil runoff can reduce *S. glomerata* growth, degenerate oyster tissues and lead to higher mortality rates (Dove and Sammut, 2007a; b). In contrast, another study observed no correlation between pH and *M. sydneyi* infection of *S. glomerata* (Anderson et al., 1994), possibly indicating that pH is more influential on the *S. glomerata* oyster host, rather than influencing the protozoan parasite itself. In addition, *S. glomerata* acclimated to acidic water through the incorporation of CO_2 into the oyster rearing tanks were shown to have a reduced tolerance to shifting salinity levels and temperature (Parker et al., 2017).

1.6.4 Nutrients

The possible role of nutrients in summer mortality disease outbreaks was first considered in the 1960's, when outbreaks of summer mortality in *C. gigas* occurred in the Matsushima Bay, Japan, a region subject to heavy eutrophication (Mori, 1979). However, since this initial evidence, the role of nutrients in oyster disease and mortality events has rarely been directly studied. Concentrations of phosphate, nitrate, nitrite, silicate and ammonium were elevated during *C. gigas* summer mortality outbreaks in Ireland and Wales, while in subsequent laboratory experiments mortality of oysters from these environments was only induced following the additions of elevated nutrient concentrations (Malham et al., 2009). To our knowledge, this is the only study to examine the role of nutrients on oyster disease in depth. Although, a previous study has shown that growing oysters in nutrient enriched seawater led to mortality rates five times greater than those oysters in non-enriched seawater (Lipovsky, 1972). In a more general context, the role of nutrients, specifically from oyster feed, on oyster larval growth and

survival has previously been reviewed (Marshall et al., 2010), with a general pattern of larvae diet strongly influencing larvae survival, as well as the need to supplement the larvae diet with protein as they progress through their life cycle (Marshall et al., 2010).

1.6.5 Translocation

While not an environmental factor, translocation is a common practice in the aquaculture industry and can unknowingly introduce pathogens to naïve areas. Examples of previous introductions of disease include marteiliosis and Dermo (Alderman, 1979; Friedman and Perkins, 1994). Marteiliosis was spread from one affected area to other parts of France and then Spain, resulting in the introduction of *M. refringens* to these areas (Alderman, 1979). Dermo was historically located in the Chesapeake Bay, but persistent introductions of infected oysters to the north-eastern USA led to the establishment of dermo in these areas (Friedman and Perkins, 1994; Ford, 1996). Often though, translocation alone is not sufficient. Environmental conditions must be favourable to the pathogen to facilitate disease establishment and progression (Ford, 1996).

1.7 The relationship between the oyster microbiome and disease

Evidence for the importance of the microbiome has been building since the term "microbiome" was first coined in 1988 (Lisansky, 1988). Arguably, the bulk of the microbiome research has been focussed on humans, with specific compositions of the human gut microbiome correlated with a number of disorders/diseases (Turnbaugh et al., 2006; Abraham and Cho, 2009; Heijtz et al., 2011). In other organisms, the microbiome influences animal behaviour and their susceptibility to pathogens (Hosokawa et al., 2008; Koch and Schmid-Hempel, 2011), for example, the microbiome of *Drosophila*

melanogaster (fruit fly) strongly drives the mating behaviour of this insect (Sharon et al., 2010). Using these examples, it is likely that the microbiome of oysters also plays a key role in oyster health, behaviour or through some contribution to the oyster disease process.

The role of the oyster microbiome in mortality outbreaks is an area of research yet to be fully explored. To date, previous research has shown that the microbiome can shift under a multitude of different stress treatments, such as translocation, starvation, temperature, infection and antibiotic stress (Green and Barnes, 2010; Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b). The microbiome also changes with different seasons (Pierce et al., 2016) and with translocation to laboratory conditions (Lokmer et al., 2016a). Additionally, while external abiotic factors can influence the microbiome, the within microbiome-interactions (between microbial organisms within a microbiome) can also play a role in bacterial community composition (Lokmer et al., 2016a) and destabilisation of this community can facilitate infection by *Vibrio* pathogens (Lokmer et al., 2016b) – this raises questions regarding the role of the oyster microbiome in disease resistance and susceptibility. Studies exploring the oyster microbiome during disease events are biased towards *C. gigas* and further towards summer mortality and the *Vibrio*-specific community.

The oyster microbiome is comprised of unique bacterial communities in each tissue, with the hemolymph bacterial community the most variable (King et al., 2012; Lokmer et al., 2016b). It has previously been proposed that destabilisation of the hemolymph microbiome can allow *Vibrio* bacteria to infiltrate the solid tissues causing a systemic infection (Lokmer et al., 2016b). There is increasing evidence that the microbiome of an organism plays an essential role in maintaining homeostasis (Shin et al., 2011; Earley

et al., 2015). For instance, in humans the microbiome maintains immune homeostasis through reduction of inflammation (Kelly et al., 2004), provides host microbial defence (Fukuda et al., 2011), assists in nutrient degradation and uptake (Turnbaugh et al., 2009) and microbiome imbalances have been linked to chronic diseases such as Crohn's disease (Frank et al., 2007). The role of the microbiome in disease dynamics is emerging as an important factor in the progression and severity of oyster diseases (Petton et al., 2015). Reduced mortality in antibiotic-treated specific-pathogen-free (SPF) oysters subsequently exposed to OsHV-1 suggests an important role for the oysters microbiome in disease dynamics (Petton et al., 2015), in particular, the *Vibrio* community in healthy *C. gigas* harbours pathogens that can induce mortality in oyster larvae (Wendling et al., 2014). Furthermore, the non-virulent *Vibrio* portion of the oyster microbiome progressively shifts towards a virulent population during the onset of summer mortality while the remaining non-virulent *Vibrio* population appears to aid in causing the disease (Lemire et al., 2015). When virulent *Vibrio* strains are injected into oysters the oyster microbiome does not become dominated by *Vibrio,* in fact, organisms from the genus *Arcobacter* become dominant (Lokmer and Wegner, 2015). Similarly, by growing the *Vibrio*-injected oysters at higher temperatures (22°C) the microbiome became more variable, with an increase in anaerobic bacteria, including members of the *Clostridia*, which were found to be a particularly large component of the microbial assemblage in dead oysters, possibly due to necrosis or anaerobic conditions (Lokmer and Wegner, 2015). From the few studies focussed on examining the *C. gigas* microbiome during a summer mortality disease outbreak, we can begin to make insights into how the native microbial community can facilitate disease progression. *C. gigas* cultivated at sites experiencing a summer mortality outbreak in Australia had a significantly different microbiome structure than specimens from sites unaffected by summer mortality (King et al., 2019a)

however, further research is required to determine the role of the whole microbiome in disease dynamics. There is evidence that shifts in the *Vibrio* community can increase the severity of disease, but it is unclear whether the whole microbial community, when stressed, provides a protective role against disease, or aids in disease progression (Thurber et al., 2009; Lemire et al., 2015; Tout et al., 2015).

To our knowledge, there has only been one study characterising the microbiome of *S. glomerata* during a disease event, with evidence that infection by *M. sydneyi* reduces the diversity of the oyster microbiome, with sequences with high homology to *Rickettsiale*-like prokaryotes highly elevated in infected oysters (Green and Barnes, 2010). Changes in the microbiome of *S. glomerata* in response to infection by *M. sydneyi* could further aid disease progression but further studies are required to examine whether mortality can be reduced in infected oysters with a more 'stable' microbiome.

The microbiome of *C. virginica* is understudied, particularly within the context of disease. To date, the culture-able bacterial community has been studied in regards to its oil degradation ability from the horizon oil spill in the Gulf of Mexico, with members of the *Pseudomonas* genus as the dominant oil-degrading isolate (Thomas et al., 2014), and the microbiome of *C. virginica* has been previously characterised using culture-independent techniques, in which the oyster gut microbiome (intestinal contents) was found to more diverse than the stomach microbiome, and the microbiome assemblage was influenced by spatial location (King et al., 2012; Chauhan et al., 2014). A recent spatiotemporal study of the *C. virginica* microbiome considered the influence of Dermo (Pierce et al., 2016). The *C. virginica* microbiome was shown to change over seasons, with the microbial

community composition significantly influenced by water temperature, but the infection and severity of Dermo disease was not found to be a significant determining factor of the microbiome (Pierce et al., 2016).

Similar to *S. glomerata* and *C. virginica,* studies of the *O. edulis* microbiome during disease events are lacking, indeed, studies characterising the healthy microbiome of *O. edulis* are also needed. To our knowledge, only one such study has examined the microbiome of *O. edulis,* with a focus on characterising the culture-able microbiome to examine shifts in the bacterial population over seasons, with isolates belonging to *Vibrio harveyi* dominant through the warmer months and *Vibrio splendidus* dominant during the colder months (Pujalte et al., 1999).

1.7.1 Oyster microbiome - future directions and challenges

Observational microbiome studies of *C. gigas* have begun to shed light on the dynamic interplay between the oyster microbiome, health, and disease. However, these studies are largely under-represented for *S. glomerata*, *C. virginica*, and *O. edulis*. It is becoming clear that applying stress to an oyster is sufficient to shift the oyster microbiome. This is seen with bacterial infection and temperature (Lokmer and Wegner, 2015), translocation (Lokmer et al., 2016b), starvation (Lokmer and Wegner, 2015), antibiotic stress (Lokmer et al., 2016a), exposure to a disease outbreak (King et al., 2019a), and parasite infection (Green and Barnes, 2010). But it is not understood how the oyster microbiome responds before, during and after an environmental disease outbreak. Understanding this dynamic is crucial for determining the microbiome contribution to disease, and whether it can 'stabilise' following stress periods. However, carrying out environmental temporal studies are particularly challenging for a number of reasons: Firstly,

in many cases the onset of disease can be very sudden and unpredictable. Secondly, holding/studying oysters in marine mesocosms (i.e. tanks or aquariums) significantly alters the oyster microbiome (Lokmer et al., 2016a) and will not be representative of an environmental outbreak. Thirdly, the oyster microbiome is highly heterogenous between replicate oysters (Lokmer et al., 2016a; King et al., 2019a). Lastly, repeated hemolymph sampling of the same individual can cause local tissue infections resulting in an over-representation of bacteria assigned to the *Tenericutes* phylum (Lokmer et al., 2016a). To overcome these challenges, environmental temporal studies will need to have a high-resolution sampling regimen to capture the mortality event, likely coupled with a large number of biological replicates to overcome the heterogeneity in the oyster microbiome.

Breeding for disease resistance is a common aquaculture practice for the mitigation of oyster disease outbreaks (Dégremont, 2011; Dove et al., 2013b). Given the likely contribution of the oyster microbiome in oyster diseases (Lemire et al., 2015; Petton et al., 2015), there is a need to determine whether breeding for disease resistance also alters the oyster microbiome composition and whether this alteration is, at least in part, responsible for disease resistance. If indeed the microbiome does play a role in disease resistance, another question is whether disease resistance oysters bred in one aquatic environment translate to another with different environmental parameters and likely microbiota. In the first instance, identifying whether disease resistance oysters have unique microbiomes will provide some insights into its protective role and stability after a disease event. Most importantly, characterising disease resistant oyster microbiomes may identify probiotic targets for the use in disease management strategies. However, as each tissue (including the hemolymph) has their own unique microbiome (Lokmer et al., 2016b), studies aiming to identify microbes

unique to disease resistant oysters might need to homogenise the oyster or use a multi-tissue approach.

Moving beyond observational microbiome studies to manipulative experiments is another key challenge. Observational studies can provide insights into which microbes are driving shifts in the microbiome and be correlated to factors such as disease resistance, but do not provide information on the functional genes playing a role in the interactome. Metagenomics has emerged as a potential but expensive replacement for 16S rRNA microbiome sequencing (Handelsman, 2004). This technique provides both observational and functional data for microbiome analysis (Quince et al., 2017). However, as extracted DNA will contain a high ratio of eukaryotic to prokaryotic DNA, enrichment of prokaryotic DNA is required before sequencing (Thoendel et al., 2016).

Once the potential functional role of these microbes has been established, another key challenge is the cultivation and manipulation of specific members of the oyster microbiome. Cultivated organisms are required to characterise the interactions between these microbes (such as those correlated to disease resistance), the host, and pathogens (Bäumler and Sperandio, 2016), and to examine the probiotic effect of these microbes (Kapareiko et al., 2011). This may identify specific genetic elements that amplify or suppress oyster diseases, allowing for the development of monitoring programs to examine the abundance of these microbes/elements in commercial stocks and breeding programs.

1.8 Conclusions

Infectious diseases afflicting oysters have remained a constant barrier for the successful growth and sustainability of oyster aquaculture industries around

the world. It is becoming increasingly apparent that the environment is an important factor driving the progression and severity of numerous oyster diseases and therefore, it is vital to consider how the environment can affect pathogen invasion and host physiology when studying oyster diseases. Oysters exist in an ever-changing environment and are constantly exposed to new challenges. In fact, the history of oyster cultivation is riddled with attempts to overcome new and existing oyster diseases (Robert et al., 2013). While the bulk of previous research has been focused on the presence of aetiological agents and their link to mortality outbreaks, future studies should begin to question why these mortality outbreaks happen, what stimulates them, and how can these mortality outbreaks be lessened by manipulating the conditions in which oysters are grown in. Furthermore, how does the microbiome fit into the disease process? Previous research has shown that the oyster microbiome can shift under a multitude of conditions, some of these conditions, such as infection stress, are able to completely replace commensal members of the microbiome with a more virulent community (Lemire et al., 2015), and microbiome destabilisation can facilitate pathogen spill over into different oyster tissues (Lokmer et al., 2016b). This virulent state can then amplify the severity of oyster diseases. Disruption of the *C. gigas* microbiome during summer mortality outbreaks is emerging as an important factor determining the progression and severity of this disease. Yet, microbiome research in other oyster species, and their role in disease, is lacking. As an oyster is exposed to a dynamic environment, the microbes they are exposed to will change, both over seasons (Wendling et al., 2014) and with climate change. Will a changing environment completely change the oyster microbiome? Will it result in more microbiome disruptions, allowing diseases to take hold more frequently? Or perhaps the oyster microbiome is more resilient than previously thought? Here we have begun to tease apart the interconnectedness of the external environment and oyster

diseases, yet it is still unclear whether the external environment acts directly on the oyster physiology and microbiome, allowing pathogens to take hold, or whether it only regulates pathogen proliferation and infection, which will cause disease regardless of the state of the oyster and its microbiome state. Answering these questions will provide vital insights into the complexity of oyster diseases and in turn, will guide management practices of oyster aquaculture to reduce the economic impact of these debilitating oyster diseases.

1.9 Acknowledgments

This work was supported by an Australian Research Council Linkage Project (LP160101785) to JRS, CJ and ML; 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 between the University of Technology Sydney and the New South Wales Department of Primary Industries.

Chapter Two

Characterisation of the Pacific oyster microbiome during a summer mortality event

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 microbial ecology.
- Approval has been granted by the publisher for inclusion of this publication in this thesis.

King, W.L., Jenkins, C., Go, J., Siboni, N., Seymour, J.R., Labbate, M. (2019). Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. *Microbial Ecology*. 2019;77(2):502-12. 10.1007/s00248-018-1226-9

Publication status: Published

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

Supplementary information is provided following the acknowledgments section

Date: 16/09/19

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

2.1 Abstract

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

2.2 Introduction

The Pacific oyster, *Crassostrea gigas*, is the most heavily cultivated oyster species globally. However, in recent years, production of *C. gigas* has been compromised by widespread and recurrent mortality events (Friedman et al., 2005; Soletchnik et al., 2005; Burge et al., 2007; Soletchnik et al., 2007;

Watermann et al., 2008; Mortensen et al., 2016). Mortalities frequently occur during the summer months, with "summer mortality" often used as an umbrella term to encompass mortalities resulting from viral and/or bacterial infection overlaid with (or precipitated by) environmental stressors (Friedman et al., 2005; Garnier et al., 2007; Malham et al., 2009).

Outbreaks of viral infections have largely been attributed to an infection by the ostreid herpesvirus (OsHV-1) or its micro-variant (OsHV-1 µvar), which affects oyster larvae, spat or juveniles (Friedman et al., 2005; Segarra et al., 2010; Mortensen et al., 2016). These OsHV-1 infections have been implicated as the causative agent of Pacific Oyster Mortality Syndrome (POMS), particularly under elevated water temperatures (Jenkins et al., 2013; Petton et al., 2013; Renault et al., 2014).

In some instances of *C. gigas* summer mortality, bacteria may also play a role with several members of the *Vibrio* genus implicated as potential disease-causing agents (Jeffries, 1982; Waechter et al., 2002; Garnier et al., 2007). These *Vibrio* infections typically target the larval and spat life stages but can also be induced in adult oysters through experimental injection challenges (Jeffries, 1982; Garnier et al., 2007). Infections with both *Vibrio* species and OsHV-1 have been previously recorded (Pernet et al., 2012) with *Vibrio* species potentially acting synergistically with OsHV-1 (Petton et al., 2015).

It is notable, however, that in many instances of summer mortality, no clear aetiological agent has been identified (Garnier et al., 2007; Go et al., 2017). For these summer mortality events (and in fact for many other oyster diseases), a number of different environmental and physiological factors, including temperature, nutrient concentrations, chlorophyll *a* levels,

turbidity, salinity, oyster growth rate and reproductive effort have been implicated as triggers for mortality events (Lipovsky, 1972; Mori, 1979; Samain et al., 2007; Soletchnik et al., 2007; Malham et al., 2009; Cotter et al., 2010). However, in most cases no single clear determinative factor(s) has been found. There is also evidence that the severity of summer mortality events is influenced by the host's genetic background and this is being exploited for disease management by breeding resistant genetic lines (Lang et al., 2010; Dégremont, 2011; Dégremont et al., 2013; Segarra et al., 2014).

Another potential factor in disease events, involves the role of the oyster microbiome. Previous studies have shown the *C. gigas* microbiome to be dynamic and responsive to external factors (Wegner et al., 2013; Lokmer and Wegner, 2015), with the microbial community responding to heat, translocation, bacterial infection and antibiotic stressors (Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016b). The microbiome is also influenced by host factors, such as the genetics of the individual oyster (Wegner et al., 2013) and the oyster life stage (Trabal et al., 2012; Trabal Fernández et al., 2014).

The role of the oyster microbiome in disease progression is an area gaining interest. Previous work has shown that while infection with OsHV-1 plays an important role in POMS, oysters pre-treated with antibiotics do not succumb to mortality, indicating that the oysters' microbiome is a factor in disease progression (Petton et al., 2015). Mortality was also correlated with low species evenness of hemolymph microbiome before translocation stress allowing *Vibrio* species to invade oyster tissues (Lokmer et al., 2016b). In addition, the resident *Vibrio* community has been observed to be replaced by virulent strains before a summer mortality disease outbreak (Lemire et al., 2015).

In the summer of 2013 to 2014, a sudden mortality event occurred in the Port Stephens estuary, New South Wales, Australia. The New South Wales Department of Primary Industries (NSW DPI) obtained oysters as a part of a structured survey to identify potential aetiolgical agents involved in this mortality event (Go et al., 2017). All oysters were found to be negative for the presence of OsHV-1 and OsHV-1 µvar and other known oyster pathogens (Go et al., 2017). Bacterial cultivation work identified numerous *Vibrio* species (*V. crassostreae*, *V. splendidus*, *V. harveyi* and *V. alginolyticus*) as being dominant in different sites, although no single clear dominant bacterium was consistently seen across the estuary (Go et al., 2017). Environmental data collected at the time indicated that sudden decreases in salinity due to rainfall and high temperature (>20°C) were likely contributors to the mortality event (Go et al., 2017). As no clear aetiological agent was found during the structured survey and to further explore this mortality event, we aimed to compare the microbiome of *C. gigas* oysters from disease-affected and disease-unaffected sites and to explore whether the oyster microbiome was influenced by spatial variation.

2.3 Methods

2.3.1 Oyster study sites and cultivation

Port Stephens is a tide-dominated estuary (Roy et al., 2001), located on the east coast of New South Wales (NSW), Australia (32°41'53.9"S 152°01'26.3"E; Figure 2.1). During a major mortality event that occurred in the austral summer of 2014 (January 6-13) *C. gigas* oysters were collected from leases where mortality was recorded. These leases were spread over seven localities within the Port Stephens estuary, of which five (Swan Bay, Karuah, Carrington, North Arm Cove and Tea Gardens) were situated in the northern part of the estuary and two (Cromartys Bay and Tilligerry Creek)

in the southern part (Figure 2.1). Among these seven localities, there were twelve sampling sites. Of these, ten sites were cultivated for commercial purposes and affected by the mortality outbreak (will be referred to as disease-affected) whereas two sites (one in Cromartys Bay and one in Karuah) were disease-unaffected wild oysters (disease-unaffected will be referred to as unaffected). The southern part of the Port Stephens estuary contains the bulk of the urban population (ABS, 2016), with only Tea Gardens in the far north eastern part of the estuary with a comparable population size (ABS, 2016), the southern sites also have a strong agricultural and mining industrial presence, particularly near Tilligerry Creek (EarthTech, 2008).

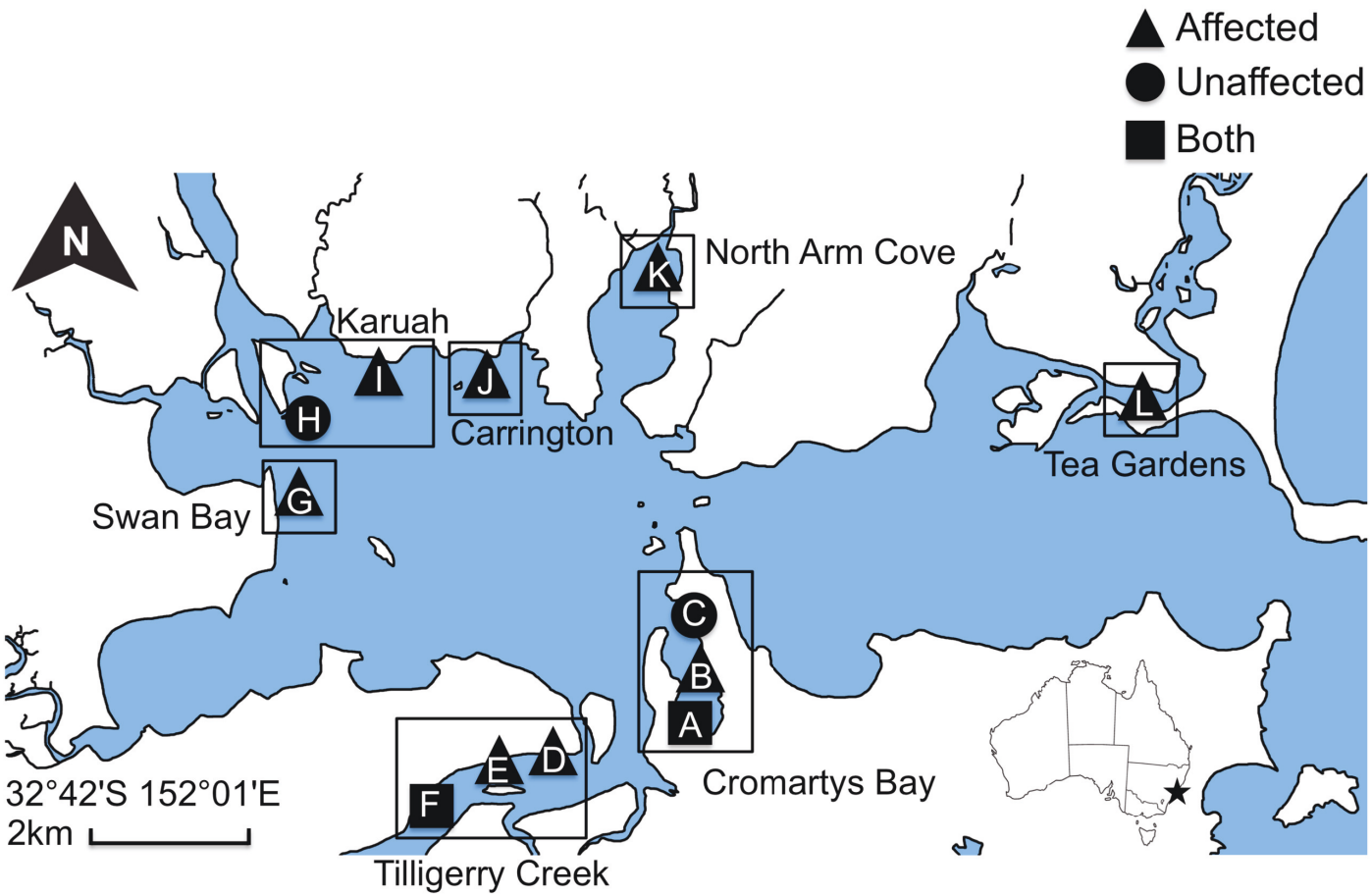


Figure 2.1. Sampling locations and sites across the Port Stephens estuary. Areas with more than one sampling site are designated as localities and are contained within boxes. Sites are numbered A through to L, which corresponds to the site numbers in Table 2.1.

Site A in Cromartys Bay contained two stocks that were either affected or unaffected by the mortality event (Figure 2.1). Site F in Tilligerry creek had two stocks with different final mortalities (10% and 99%). Due to the low mortality for this stock (10%) and the significantly different microbial assemblage (One-way ANOSIM using five samples per stock: $p = 0.0067$; $R = 0.61$ with 9999 permutations) to the 99% mortality stock, it was treated as an unaffected stock. The oyster cultivation conditions at each site (where known) are summarised in Table 2.1. Diploid oysters were cultivated at all sites, with the exception of one site in Tilligerry creek, where triploid oysters were cultivated. Oysters were predominately grown in trays except for those in North Arm Cove and Karuah (disease-affected site), which were grown in long line baskets. Cultivated oysters were sourced from a Tasmanian hatchery, a Port Stephens-based hatchery or, wild-caught oyster seed or were wild non-cultivated oysters.

Table 2.1 – The oyster stock source, ploidy status and cultivation method (where known) for oysters at all study sites

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

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

2.3.2 Oyster sample processing and DNA extraction

Juvenile oysters were collected from each site and transported back to the laboratory in iced containers as previously described (Go et al., 2017), where they were stored at -80 °C prior to analysis. Five samples from each sampling site were thawed and scrubbed with a hard-bristled brush under running water to remove any remaining mud and debris on the outer shell. Samples were then shucked using sterile shucking knives and immediately placed into sterile petri dishes. Approximately 25 mg of adductor muscle tissue was dissected and removed using sterile scalpel blades. Hemolymph collected *via* aspiration of the adductor muscle sinuses is frequently used to examine the bacterial population circulating within oysters (Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b; Vezzulli et al., 2017) however, the use of frozen oysters in this study precluded aspiration of the hemolymph via syringe. Therefore, in line with the approaches used in several previous studies (Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b) aseptically dissected adductor muscle tissue containing hemolymph sinuses (Gagnaire et al., 2008) was used to capture the fluid contained within.

DNA was extracted from the dissected adductor muscle using the Qiagen DNeasy blood and tissue kit (catalogue: 69506), as per the manufacturer's instructions. Extracted DNA was then amplified using PCR targeting the ribosomal 16S rRNA V1-V3 region using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') primer pair (Lane, 1991; Turner et al., 1999). The PCR cycling conditions were as follows: 94°C for two minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Amplicons were sequenced using the Illumina MiSeq platform (version 3, 2 x 300bp) at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney,

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

2.3.3 Data analysis

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME version 1.9.1) pipeline (Caporaso et al., 2010). Briefly, paired-ended DNA sequences were joined with join_paired_ends.py, OTUs were defined at 97% sequence identity using UCLUST (Edgar, 2010) using open-reference picking, and taxonomy was assigned against the Greengenes database (version 13/08/2013) (McDonald et al., 2012) using the RDP classifier (Wang et al., 2007). Chimeric sequences were then identified using ChimeraSlayer. Mitochondrial, chloroplast and chimeric sequences were filtered out of the dataset. Remaining sequences were rarefied to allow for even coverage across all samples. Relative abundance per sample was calculated and those OTUs with a relative abundance below 0.1% were filtered from the dataset. Alpha diversity indices, including Shannons Index, Chao1 and Shannons Index/log (observed species) were used to calculate species diversity, species richness and species evenness respectively, using QIIME (Caporaso et al., 2010).

2.3.4 Statistical analyses

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

used with a non-metric multidimensional scaling (nMDS) analysis. To determine the statistical significance of apparent patterns identified by nMDS a one-way ANOSIM with 9999 permutations was used. To identify which OTUs contribute to the greatest differences between locations and/or disease states, SIMPER analysis was used. To determine whether specific OTUs (such as those OTUs with the highest summed abundance across all samples, and those OTUs identified as determinants of difference between samples using SIMPER) were significantly different between disease states, a Kruskal-Wallis ANOVA was used. All of these analyses were carried out using PAST (Hammer et al., 2001). To determine whether an OTU was significantly elevated in a particular disease state at Site A, the group_significance.py script using the default analysis (Kruskal Wallis ANOVA) was used in QIIME.

2.4 Results

2.4.1 Sequence read depth and rarification

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

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

We observed a high level of within-site variation in the composition of the *C. gigas* microbiome (Figure 2.2). For instance, unaffected oyster microbiomes from Site A (Table 2.1), exhibited low similarity (defined by a

low median) between replicates (Median ± Standard Deviation; 0.13 ± 0.10), while disease-affected replicates in Tilligerry creek (Site D; 98% mortality) had the lowest variability (defined by a low standard deviation) between individuals (0.27 ± 0.05). At Cromartys Bay site A, disease-affected oyster microbiomes had significantly less inter-oyster variability than the unaffected oysters (Kruskal-Wallis ANOVA, $p = 0.005$; 0.33 ± 0.09 and 0.13 ± 0.10 respectively; Supplementary Table 2.1), this was also observed at Site F in Tilligerry Creek ($p = 0.007$; disease-affected 0.36 ± 0.097 and unaffected 0.21 ± 0.075). There was no significant difference when comparing variation between those oyster microbiomes in the north versus the south ($p = 0.29$). However, disease-affected microbiomes in the north had significantly less similarity between samples than those in the south ($p = 0.009$; 0.28 ± 0.089 and 0.34 ± 0.149 respectively). Differences in the local environment could potentially explain this difference in similarity, as the southern part of the estuary is largely urbanised and has a strong agricultural and mining presence (ABS, 2016).

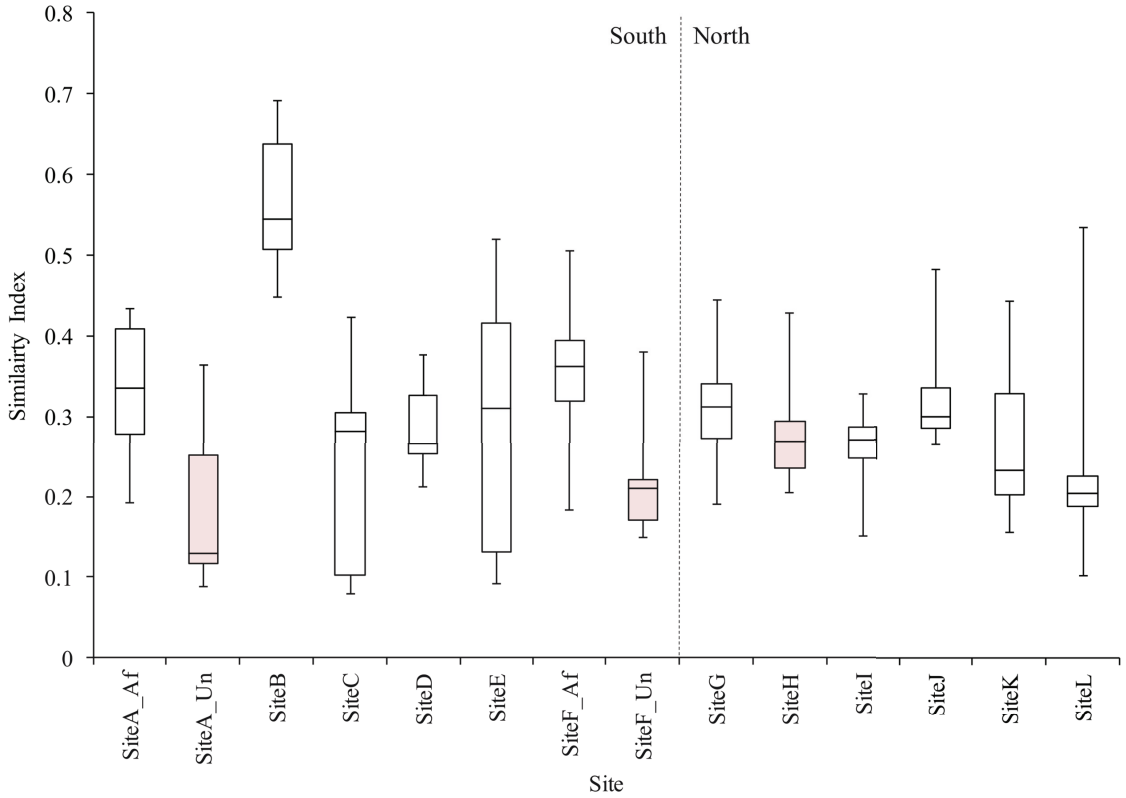


Figure 2.2. Box and whisker plot of similarity indices calculated with a Bray-Curtis dissimilarity index. Each site code corresponds to those values listed in Table 2.1. As sites A and F contained both disease-affected and unaffected oyster stocks, these sites listed with the suffix '_Af" are disease-affected stocks, while those with '_Un' are unaffected stocks. Each site has 5 replicate oysters. Northern and southern samples are separated by the black vertical line. Disease-unaffected sites are shaded red.

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

We sought to examine whether the microbial assemblage of oyster microbiomes were influenced by disease-state, spatial location and stock source. The *C. gigas* microbiome composition significantly differed according to spatial location and disease state (Figure 2.3). Oyster samples taken from sites experiencing no mortalities had a significantly different microbiome composition than samples from disease-affected sites in the north ($p = 0.0002$; $R = 0.26$) and south ($p = 0.0001$; $R = 0.21$). In addition, oysters collected from sites exhibiting high mortalities within the southern region of the estuary exhibited a microbiome composition that differed significantly from the disease-affected oysters in the northern region ($p = 0.0001$; $R = 0.48$; Figure 2.3), with species diversity ($F_{(1, 48)} = 4.16$; $p = 0.047$;) and richness ($F_{(1, 48)} = 14.15$; $p = 0.00046$) also differing between the northern and southern regions of the estuary, while species evenness did not differ between regions ($F_{(1, 48)} = 1.07$; $p = 0.31$) (Supplementary Table 2.1). Disease-affected hatchery sourced oysters also had a significantly different microbial assemblage to disease-affected wild sourced oysters ($p = 0.0052$; $R = 0.19$).

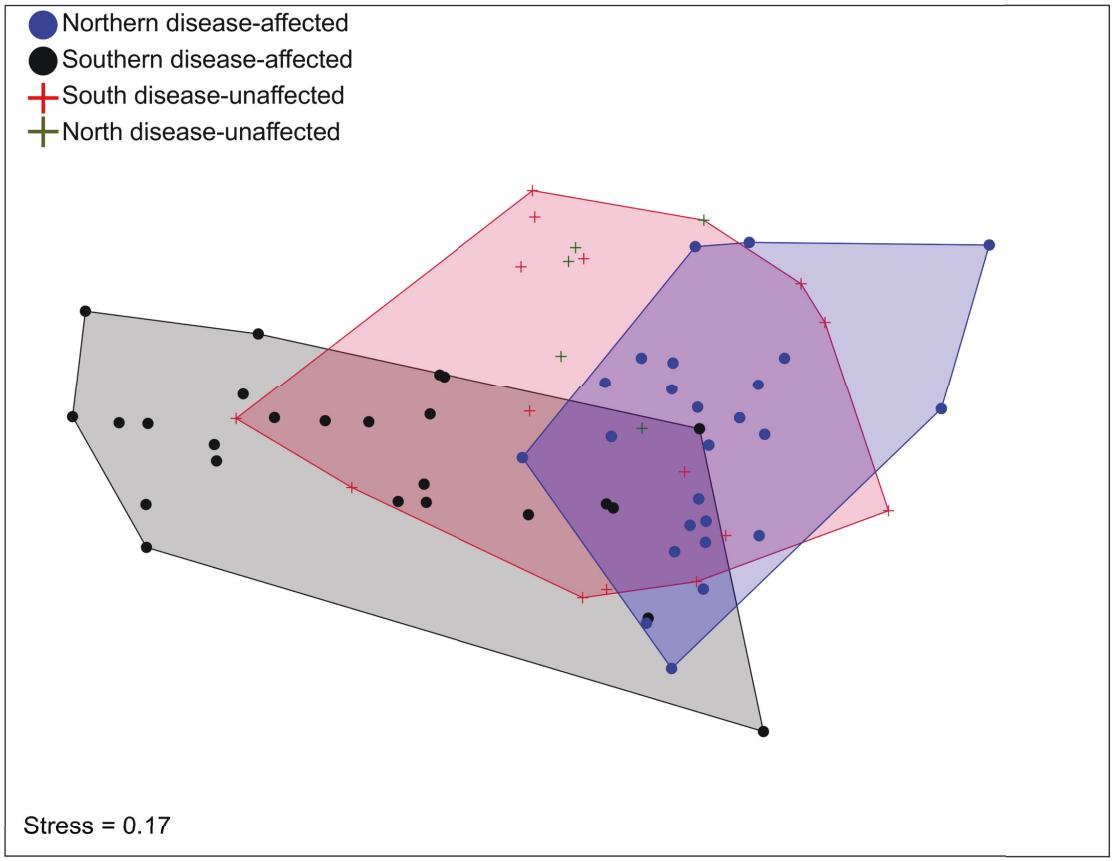


Figure 2.3. nMDS plot showing spatial and disease state separation. Samples separate spatially based on region, as well as by their disease state. Axes 1 and 2 are plotted.

As we observed a significant difference between the disease-affected and disease-unaffected microbiome composition, we sought to examine persistent ('core') bacteria unique to these disease-states and across the estuary as a whole (Supplementary Table 2.2). We identified 24 core members, of which 1 core OTU, an unidentified member of the *Mollicutes* class (OTU 89399), was unique to disease-affected microbiomes, while 8 OTUs were unique to disease-unaffected microbiomes (Supplementary Table 2.2). BLASTing the representative sequence for OTU 89399 against the NCBI database identified it as an uncultured bacterium previously observed in *C. gigas* and another oyster species, *Saccostrea glomerata* (both BLAST hits were E-value: 0, Identity: 99%) (Green and Barnes, 2010; Fernandez-Piquer et al., 2012). We then sought to examine which OTUs were driving the microbiome composition difference between disease states, and between spatial location. According to SIMPER analysis, the microbiomes of all unaffected samples (20 samples) were found to be 86.1% dissimilar to disease-affected samples in the south (25 samples) and 80.1% dissimilar to disease-affected samples in the north (25 samples; Supplementary Table 2.3).

Due to the spatial separation of oyster microbial communities between the northern and southern regions of the estuary, patterns in the relative abundance of dominant OTUs within each region were examined separately to determine whether these OTUs were associated with disease-affected or unaffected oysters. In the northern region, the top five dominant OTUs from all sites belonged to the *Brachyspiraceae* family (OTU 32677), *Mycoplasma* genus (OTU 38764), *Mycoplasma* genus (OTU 3538), *Mollicutes* class (OTU 89399) and the *Alphaproteobacteria* class (OTU 556), with these OTUs representing 28.8%, 9.3%, 3.2%, 3.2% and 2.7% of the total community respectively. OTUs assigned to the *Mycoplasma* genus (OTU

38764), *Mycoplasma* genus (OTU 3538) and the *Mollicutes* class (OTU 89399) were more abundant in disease-affected samples ($p = 0.039$; $p = 0.044$ and $p = 0.0097$ respectively), while the relative abundance of an unidentified member of the *Alphaproteobacteria* class (OTU 556) and *Brachyspiraceae* family (OTU 32677) was uniform across all samples ($p = 0.73$ and $p = 0.16$ respectively).

In the south, the five most dominant OTUs across all sites were members of the *Brachyspiraceae* family (OTU 32677), *Spirochaetia* class (OTU 20129), *Mycoplasma* genus (OTU 38764), *Pseudoalteromonadaceae* family (OTU 18290) and the *Alphaproteobacteria* class (OTU 556), with these OTUs representing 10.8%, 8.3%, 7.8%, 5.2% and 3.3% of sequences in the south. OTUs assigned to the *Pseudoalteromonadaceae* (OTU 18290) and *Brachyspiraceae* family (OTU 32677) were elevated in the disease-affected samples ($p = 0.48$ and $p = 0.00055$ respectively), while the remaining dominant OTUs were found in both unaffected and disease-affected samples.

2.4.4 Cromartys Bay C. gigas microbiomes shift in accordance with disease state

One location in Cromartys Bay (Site A) contained both disease-affected (75% mortality) and unaffected (0% mortality) oyster stocks (Figure 2.1). Due to the apparent role of spatial location in shaping the microbiome, and to account for any potentially confounding external factors such as cultivation method, hatchery source and unique local microenvironments, we focused on Site A to examine differences in the microbiome of unaffected and disease-affected oysters. While Site F in Tilligerry Creek also had oyster stocks that were disease-affected and unaffected, this site was the only studied site to grow triploid oysters and information about the cultivation methods used at this site are unknown (Table 2.1). Because ploidy status and

differing cultivation methods play a role in mortality outbreaks (Pernet et al., 2012), Site A was selected as the preferred site for comparative analysis.
All oysters from Site A were cultivated in trays from hatchery spat that were acquired from the same source, thus minimising any potential differences in the microbiome caused by host genetics or cultivation method. While these two sample groups displayed no difference in species diversity ($p = 0.46$) or evenness ($p = 0.84$) and only a marginally significant difference with species richness ($F_{(1, 8)} = 8.5$; $p = 0.019$), they exhibited significantly different microbiome composition ($p = 0.0073$; $R = 0.58$; Figure 2.4; Supplementary Figure 2.1; Supplementary Table 2.1), and were 86.5% dissimilar to each other according to a SIMPER analysis. This dissimilarity identified with a SIMPER analysis was primarily driven by an over-representation of OTUs assigned to the *Pseudoalteromonadaceae* family (OTU 18290), *Bacteroidales* order (OTU 27418), JTB215 family (of the order *Clostridiales*; OTU 100999), *Bacteroidales* order (OTU 86667), *Paludibacter* genus (OTU 31616) and the *Bacteroides* genus (OTU 94495) in the disease-affected samples ($p = 0.0090$; $p = 0.0082$; $p = 0.0053$; $p = 0.0053$; $p = 0.019$; $p = 0.034$ respectively; Supplementary Table 2.4).

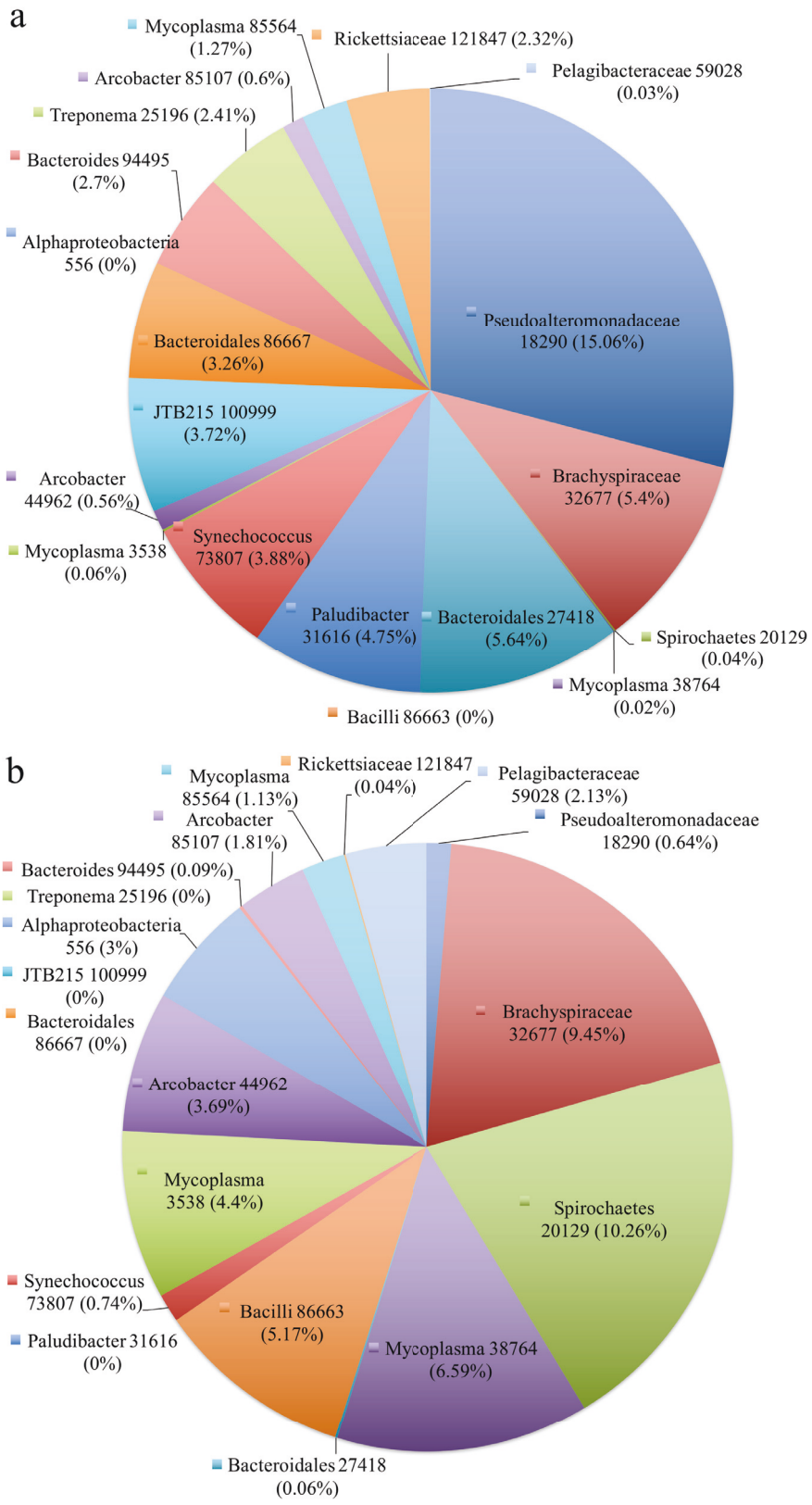


Figure 2.4. Average abundance of Pacific Oyster microbiomes at Cromartys bay Site A. a) represents disease-affected samples, while b) shows unaffected samples. A large shift in the microbiome is evident in disease-affected samples. OTUs representing less than 0.1% relative abundance were filtered out of the data set and only the top 20 OTUs are displayed, representing 51.7% and 49.2% of the averaged relative abundance community in a) and b) respectively. Numbers in brackets on the pie chart represent the average relative abundance for each pie slice.

To examine whether there were significant changes in the relative abundance of OTUs between disease states, we used the group_significance.py analysis with the default Kruskal-Wallis ANOVA parameters, of these, we chose OTUs assigned to the genus and species level for further examination. OTUs assigned to the *Paludibacter* genus (OTU 31616; p = 0.0070), *Bacteroides* genus (OTU 94495; p = 0.022), *Treponema* genus (OTU 25196; p = 0.0050), *Arcobacter* (OTU 4188; p = 0.011), *Vibrio harveyi* (OTU 67592; p = 0.0060) and *Vibrio* genus (OTU 122517; p = 0.015) were significantly elevated in disease-affected oysters (Table 2.2), while OTUs assigned to the *Pseudoalteromonas* genus (OTU 38778; p = 0.016), *Mycoplasma* genus (OTU 109572; p = 0.030), *Costertonia aggregata* (OTU 16511; p = 0.025) and the *Amphritea* genus (OTU 69264; p = 0.05) were significantly overrepresented in disease-unaffected samples (Table 2.2).

Table 2.2 – Significantly elevated OTUs (Kruskal-Wallis ANOVA; $p < 0.05$) between disease states at Site A as well as their average abundance. OTUs assigned down to the genus or species level were chosen.

OTU	**Diseased abundance (%)**	**Healthy abundance (%)**
Bacteroides 94495	2.70	0.087
Paludibacter 31616	4.75	0
Costertonia aggregata 16511	0	0.18
Formosa crassostrea 88998	0.024	0
Tenacibaculum 125471	0	0.029
Fusibacter 119674	0.028	0
Fusobacterium 6434	0.57	0
Psychrilyobacter 42830	0.038	0
Nautella 120088	0.52	0.027
Octadecabacter antarcticus 25878	0.15	0
Desulfotalea 96648	0.069	0
Arcobacter 4188	1.70	0
Amphritea 69264	0	0.099
Pseudoalteromonas 38778	0	0.56
Pseudoalteromonas piscicida 110272	0	0.036
Vibrio harveyi 67592	0.15	0
Vibrio 122517	0.22	0
Treponema 25196	2.41	0
Mycoplasma 109572	0	0.36

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

2.5 Discussion

2.5.1 Oyster microbiomes have large within-site heterogeneity

Oyster samples examined in this study displayed a high degree of within-site microbiome variability, which is consistent with previous work that has demonstrated substantial inter-oyster heterogeneity in microbiome composition (Wegner et al., 2013). A previous study has shown that the rare specialist community is governed by the genetics of individual oysters (Wegner et al., 2013), which may be responsible for the variability between replicate oysters. However, as we do not have any information pertaining to the population structure of these oysters we cannot account for the genetic diversity between wild and hatchery sourced oysters. Despite the inter-oyster within-site oyster microbiome heterogeneity, the variability between

northern and southern sites and disease state were larger. Nonetheless, the high variability between individual microbiomes may be the reason for the low power for many of the statistical tests performed here, suggesting that future studies examining oyster microbiomes might need to account for this with increased replicates.

2.5.2 Oyster microbiomes are influenced by spatial location

The high degree of location specific difference between diseased samples in the northern and southern regions of the Port Stephens estuary was arguably surprising given that the estuary is only approximately 5km wide. In contrast to our observations, little to no spatial heterogeneity in the composition of oyster microbiomes was observed across the Wadden Sea in Northern Europe, which spans an area of approximately 200km (Lokmer et al., 2016a). A previous study indicates that genetics plays a minor role in explaining the variability between individual oyster microbiomes (Wegner et al., 2013), in agreement, the oyster microbiomes of hatchery sourced disease-affected oysters (primarily from southern sites) were found to be significantly different to wild sourced disease-affected oyster microbiomes (primarily northern sites), therefore it is possible that genetics play a small role in explaining the spatial separation of oyster microbiomes in this study. However, further research is required to isolate the importance of these variables on the disease-affected oyster microbiome.

2.5.3 Within site comparison of microbiomes between disease states

As the site at Cromartys Bay (Site A) contained oysters from both disease-affected and unaffected trays, we could remove the confounding effects of spatial variation to examine the within-site differences in the microbiome of disease-affected and disease-unaffected oysters. At Site A, OTUs belonging to *V. harveyi* and an unidentified member of the *Vibrio* genus (Table 2.2)

were found to be significantly more abundant in disease-affected samples. This is consistent with previous studies that have implicated the *Vibrio* community for their role in oyster disease outbreaks (Garnier et al., 2007; Lemire et al., 2015). *C. gigas* experimentally infected with a virulent *Vibrio* strain show an increase in *Vibrio* abundance in the microbiome, but the relative abundance remains low, despite significant disease symptoms and shifts in the rest of the microbiome (Lokmer and Wegner, 2015). Similarly, a small non-significant fold increase (~1.4) of *Vibrio* spp. abundance in heat stress corals is sufficient to cause large microbiome metabolic shifts (Thurber et al., 2009). Replacement of non-virulent strains with virulent strains has been documented in *C. gigas* during a summer mortality disease event (Lemire et al., 2015) indicating that rare *Vibrio* species can disrupt the microbiome to a disease susceptible state. While 16S rRNA is often unable to provide sufficient resolution to observe shifts within *Vibrio* populations, we observed increases in *V. harveyi*. This species has previously been identified as a *C. gigas* pathogen (Saulnier et al., 2010), and its increase in relative abundance in our sequencing data is consistent with the higher *Vibrio* bacterial counts in disease-affected samples from this specific site (Go et al., 2017). Furthermore, a prior study has demonstrated that the hemolymph microbiomes of *Vibrio*-infected *C. gigas* showed an increase in bacteria from potentially pathogenic genera, such as *Photobacterium,* and bacteria belonging to *Bacteroidia*, *Clostridia*, *Propionigenium*, *Vibrio*, *Arcobacter* and *Mollicutes* (Lokmer and Wegner, 2015). It is notable that similar increases in bacteria belonging to these groups were observed in this study, with an unidentified member of the *Mollicutes* identified as being a core member of disease-affected microbiomes. While it is not possible to determine to what extent these *Vibrio* OTUs caused this oyster mortality event, our observations of their elevated abundance in diseased oysters and evidence from previous work (Garnier et al., 2007; Lemire et al., 2015;

Lokmer and Wegner, 2015) points towards a potential role in infection or opportunistic colonisation.

Sewage associated bacteria were found to be significantly elevated in disease-affected samples at Site A, in particular the *Paludibacter* genus 31616 was found to be completely absent in the unaffected samples, while those assigned to the *Bacteroides* genus 94495 were significantly elevated in disease-affected samples at both Site A and in the southern region as a whole. The *Paludibacter* genus has been associated with animal waste (Ueki et al., 2006; Newton et al., 2011), while the *Bacteroides* genus is found to be heavily abundant in the human gastrointestinal tract (GIT) (reviewed by (Bäckhed et al., 2005)) and can be used as an indicator for human faecal contamination (Kreader, 1995; Newton et al., 2011). This over-representation of sewage/faecal associated OTUs in the southern site may be explained by the geographical features of the Port Stephens estuary, with Tilligerry creek having a strong agricultural and mining industrial presence on the creek, as well as being exposed to effluent off-flow from septic systems during periods of high rainfall (EarthTech, 2008). Interestingly, there was a small rainfall event of 12.6mm approximately eight days before the start of the first mortalities at Cromartys Bay. Tilligerry creek flows out towards the northeast (EarthTech, 2008), as Cromartys Bay sits at the mouth of Tilligerry creek, it is possible that water entering Cromartys Bay influenced the bacterial communities in that bay as well. Due to their elevation in only the disease-affected samples, it may be possible that unaffected oysters had greater capability to flush out these bacteria from their tissues.

2.6 Conclusion

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

2.7 Acknowledgments

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

2.8 Supplementary information

Supplementary Table 2.1. Comparisons of alpha and beta diversity between disease-states and locations.

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

KS represents a Kruskal-Wallis ANOVA

SIM represents a one-way ANOSIM

ANO represents a one-way ANOVA

SA represents Cromartys bay Site A

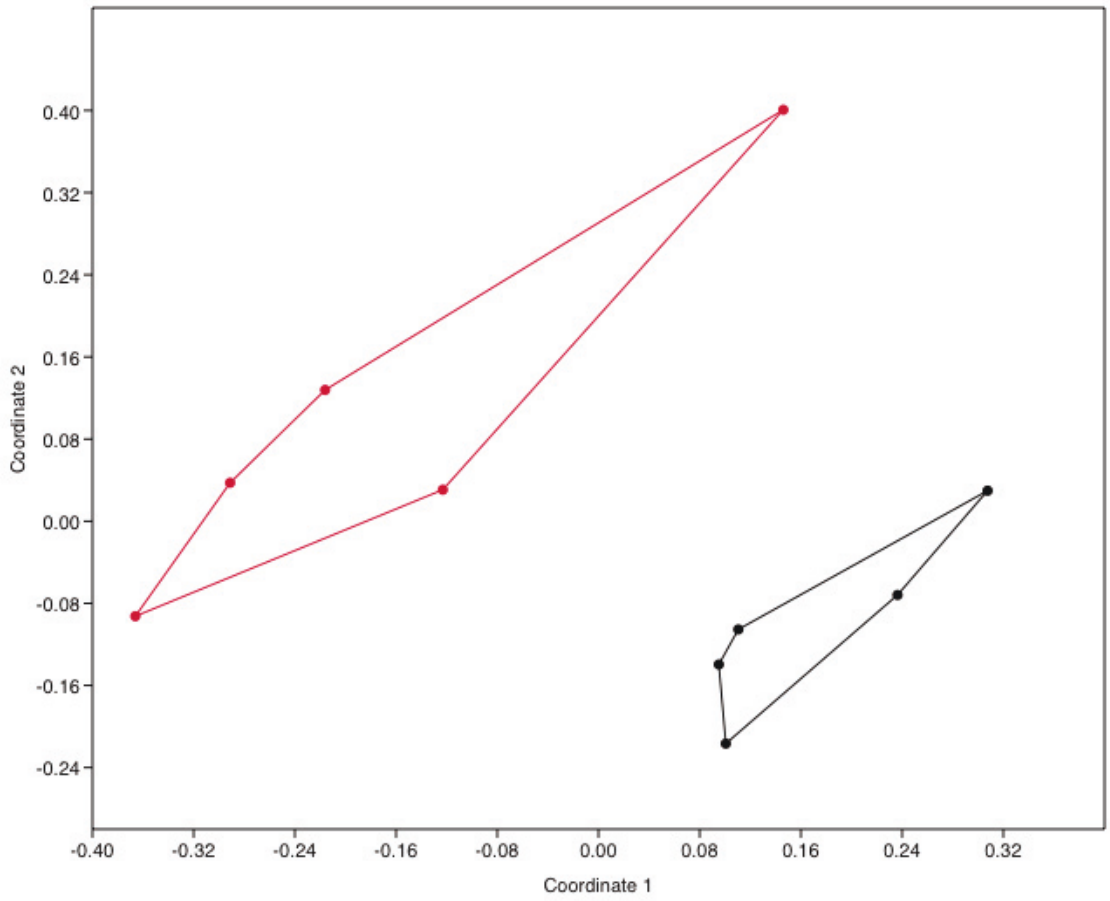
SF represents Tilligerry creek Site F

Supplementary Table. 2.2. Core microbiome of disease-affected and disease-unaffected oyster microbiomes. Unique OTUs are those OTUs exclusively found in that disease state, while shared OTUs are those found in both disease states. Analysis was run using the panbiom.py script (Kahlke, 2017), with a core OTU member defined as an OTU present in 80% of samples.

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

Supplementary Table. 2.3. SIMPER analysis of disease-affected oyster microbiomes (northern or southern region) compared to disease-unaffected oyster microbiomes. The top 10 OTUs are displayed with their dissimilarity contribution and mean transformed representation.

OTU	Dissimilarity (%)	North disease-affected mean	Disease-unaffected mean
Brachyspiraceae 32677	2.70	4.93	3.6
Mycoplasma 38764	2.65	2.42	1.7
Spirochaetes 20129	1.76	0.45	0.90
Mycoplasma 3538	1.51	1.41	0.85
Pseudoalteromonadaceae 18290	1.37	0.47	1.46
Mollicutes 89399	1.37	1.18	0.47
Mycoplasma 78519	1.25	1.11	0.76
Mycoplasma 85564	1.23	0.85	0.83
Polynucleobacter 20780	1.21	1.07	0.93
Alphaproteobacteria 556	1.02	0.78	0.38
OTU	Dissimilarity (%)	South disease-affected mean	Disease-unaffected mean
Brachyspiraceae 32677	2.50	1.68	3.6
Mycoplasma 38764	2.08	1.28	1.7
Spirochaetes 20129	2.03	1.01	0.90
Pseudoalteromonadaceae 18290	1.67	1.76	1.46
Bacteroidales 50223	1.35	1.42	0.14
Mycoplasma 85564	1.25	1.16	0.83
Bacteroidales 27418	1.03	1.09	0.04
Bacteroides 94495	1.01	1.01	0.05
Mycoplasma 3538	1.00	0.59	0.85
Alphaproteobacteria 556	0.96	0.50	0.38



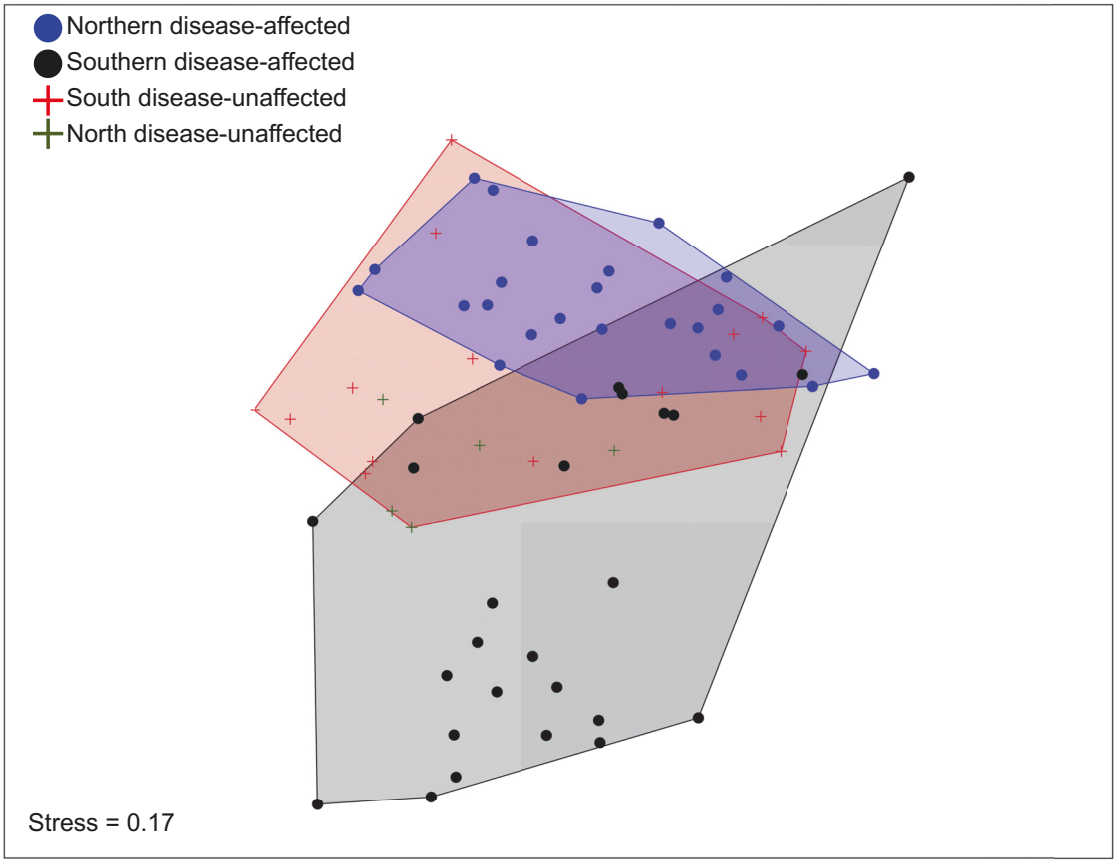
Supplementary Figure 2.1. nMDS plot showing disease state separation between *C. gigas* microbiomes at Cromartys Bay Site A. Disease-affected samples are represented as black circles and are outlined in black. Unaffected samples are represented as red circles and are outlined in red. Samples separate based on disease state. Stress 0.084

Supplementary Table. 2.4. SIMPER analysis of Cromartys bay Site A. The top 10 OTUs are displayed with their dissimilarity contribution and mean transformed representation in either disease-affected or disease-unaffected samples

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

*Of the order *Clostridiales*

^Significantly more abundant with a Kruskal-Wallis ANOVA ($p < 0.05$)



Supplementary Figure 2.2. nMDS plot showing spatial and disease state separation. Samples separate spatially based on region, as well as by their disease state. Axes 1 and 3 are plotted.

Preamble for Chapters Three, Four, and Five

The work presented in chapter two produced the first observations of the oyster microbiome in relation to summer mortality disease outbreaks, this work observed a significant effect of disease exposure on the oyster microbiome. Furthermore, the microbiome was found to be spatially influenced, and members of the *Vibrio* genus were found to be significantly elevated in those microbiomes from disease-affected sites. As such, chapter two of this thesis opened up a number of novel research questions for further scrutinisation. These research questions are outlined below:

Research question one: a microbiome comparison of oysters from the same site showed a significant difference in the microbiome composition in accordance with disease exposure. However, it is unclear why two oyster stocks at the same site had significantly contrasting levels of mortality. One such explanation could be that the disease-unaffected oyster stocks were resistant to disease, and/or they had a different microbiome composition prior to the disease outbreak. To investigate whether breeding for disease-resistance (and therefore the genetics of the oyster) significantly influenced the oyster microbiome, oysters with varying degrees of disease resistance were acquired from the Australian Seafood Industries (ASI) and subject to microbiome analysis. This research questions corresponds to chapter three of this thesis.

Research question two: spatial location was found to be a significant determinant of the oyster microbiome across the Port Stephens estuary. However, it was unclear whether this influence was driven by disease exposure, genetics, local environment, or a mixture of these variables. In an attempt to further explore the factors that govern the structure of the oyster

microbiome, a study was designed to analyse oyster microbiomes from six different oyster farms across New South Wales, with a specific emphasis on using a multi-tissue targeted approach, to examine microbiome heterogeneity within, and between oysters, with a specific emphasis to identify conserved patterns across all locations and oyster tissues. This research question corresponds to chapter four of this thesis.

Research question three: members of the *Vibrio* genus were found to be significantly elevated in disease-affected oyster microbiomes. This observation corresponds to previous studies identifying a role for *Vibrio* bacteria in oyster disease outbreaks. Due to the importance of *Vibrio* bacteria in oyster diseases, and because current sequencing technologies have poor taxonomic resolution for this genus, an assay was developed to improve the taxonomic resolution of the *Vibrio* genus when using high-throughput sequencing techniques. This assay was applied to a laboratory-induced disease outbreak to examine the *Vibrio* community composition during the outbreak. This research questions corresponds to chapter five of this thesis.

Chapter Three

Variability in the composition of Pacific oyster microbiomes across oyster family lines exhibiting different levels of susceptibility to OsHV-1 µvar disease

Chapter Three - 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 frontiers in microbiology.
- The publication is open access and no approval from the publisher is necessary.

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. (2019). Variability in the composition of Pacific Oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 μvar disease. *Frontiers in Microbiology* 10, 473. 10.3389/fmicb.2019.00473

Publication status: Published

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

Supplementary information is provided following the acknowledgments section

Date: 16/09/19

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

3.1 Abstract

Oyster diseases are a major impediment to the profitability and growth of the oyster aquaculture industry. In recent years, geographically widespread outbreaks of disease caused by ostreid herpesvirus-1 microvariant (OsHV-1 µvar) have led to mass mortalities among *Crassostrea gigas*, the Pacific Oyster. Attempts to minimise the impact of this disease have been largely focussed on breeding programs, and although these have shown some success in producing oyster families with reduced mortality, the mechanism(s) behind this protection is poorly understood. One possible factor is modification of the *C. gigas* microbiome. To explore how breeding for resistance to OsHV-1 µvar affects the oyster microbiome, we used 16S rRNA amplicon sequencing to characterise the bacterial communities associated with 35 *C. gigas* families, incorporating oysters with different levels of susceptibility to OsHV-1 µvar disease. The microbiomes of disease-susceptible families were significantly different to the microbiomes of disease-resistant families. OTUs assigned to the *Photobacterium*, *Vibrio*, *Aliivibrio*, *Streptococcus* and *Roseovarius* genera were associated with low disease resistance. In partial support of this finding, qPCR identified a statistically significant increase of *Vibrio*-specific 16S rRNA gene copies in the low disease resistance families, possibly indicative of a reduced host immune response to these pathogens. In addition to these results, examination of the core microbiome revealed that each family possessed a small core community, with OTUs assigned to the *Winogradskyella* genus and the *Bradyrhizobiaceae* family consistent members across most disease-resistant families. This study examines patterns in the microbiome of oyster families exhibiting differing levels of OsHV-1 µvar disease resistance and reveals some key bacterial taxa that may provide a protective or detrimental role in OsHV-1 µvar disease outbreaks.

3.2 Introduction

The Pacific oyster, *Crassostrea gigas* is a globally cultivated oyster species, but the cultivation of this species has been increasingly impacted by disease events (Azéma et al., 2015). These disease events are largely caused by viral and bacterial aetiological agents (Friedman et al., 2005; Garnier et al., 2007; Segarra et al., 2010; King et al., 2019b), but in some instances no clear aetiological agent is identifiable (Go et al., 2017; King et al., 2019a). A major pathogen of *C. gigas* is the ostreid herpesvirus 1 (OsHV-1), and its micro variant form (OsHV-1 μvar) (Davison et al., 2005; Segarra et al., 2010). This virus has caused severe mortality outbreaks over the last two decades (Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Mortensen et al., 2016), with some outbreaks resulting in over 90% mortality and leading to the death of many millions of oysters (ASI, 2015).

To combat the impact of OsHV-1 μvar, a variety of approaches including modifying husbandry practises (e.g. increased oyster cultivation height) and breeding disease resistant oysters have been applied, with varying degrees of success (Dégremont, 2011; Paul-Pont et al., 2013; Whittington et al., 2015). Breeding programs generally involve breeding oyster genetic lines that have greater survival rates following exposure to OsHV-1 (Dégremont, 2011; 2013; Dégremont et al., 2015b; Camara et al., 2017). While these breeding programs have shown some success, resistant families still experience varying degrees of mortality (juvenile oysters 5-19%; larvae up to 86%) (Dégremont, 2011; Dégremont et al., 2013; Dégremont et al., 2016b), and the mechanism(s) underpinning resistance are often not easily distinguishable.

A number of studies have characterised the physiological and immunological factors driving OsHV-1 and OsHV-1 μvar resistance in selectively bred

oysters (Sauvage et al., 2009; Dégremont, 2011; 2013; Azéma et al., 2015; Dégremont et al., 2015a; Dégremont et al., 2016a; Dégremont et al., 2016b). Factors such as increased oyster size and weight are associated with increased resistance to infection, but why this occurs is currently unclear (Dégremont, 2013; Dégremont et al., 2015b). Other studies have determined that resistant oysters have a greater capacity to clear OsHV-1 from their tissues and supress virus replication (Dégremont, 2011; Segarra et al., 2014). When examined from an immunologic perspective, resistant oysters appear to have greater capacity to induce autophagy genes when infected by OsHV-1 compared to susceptible oysters (Moreau et al., 2015).

Another contributing factor that has received less attention is the oyster microbiome. A previous study has shown that despite being positive for OsHV-1 µvar, antibiotic-treated oysters displayed significantly reduced mortalities in comparison to untreated oysters (Petton et al., 2015). Furthermore, the total bacterial load, including the *Vibrio* community, is significantly elevated following OsHV-1 µvar infection and this elevation is necessary to cause mortality (De Lorgeril et al., 2018). *Vibrio* bacteria are commonly isolated from OsHV-1 infected oysters (Segarra et al., 2010; Garcia et al., 2011; Jenkins et al., 2013; Keeling et al., 2014), with a recent study concluding that OsHV-1 µvar infection causes immune-suppression of the oyster host, allowing opportunistic bacteria (including *Vibrio* species) to infect the oyster (De Lorgeril et al., 2018). In other organisms, studies have implicated the host microbiome as modulating the immune system, suggesting it is critical in host defence and overall health (reviewed by (Shreiner et al., 2015)) and in influencing host behaviour (Shin et al., 2011).

To better understand how breeding for OsHV-1 µvar disease resistance affects the *C. gigas* microbiome and to elucidate whether specific taxa are

associated with susceptibility and resistance, we examined the microbiome of 35 *C. gigas* families with varying degrees of disease-resistance. To remove the confounding effects of time and location, these oysters were deployed at a single location and sampled at the same time. In addition, the comparison of distinct *C. gigas* families provided the opportunity to determine whether they harboured distinct microbial community assemblages and whether persistent bacterial taxa (core microbiome) common across the families could be identified.

3.3 Methods

3.3.1 Sources and sampling of C. gigas

Australian Seafood Industries (ASI) is an oyster aquaculture industry-owned company that since the first OsHV-1 μvar outbreak in 2010 has been breeding *C. gigas* families for OsHV-1 μvar disease resistance through field exposure. In 2016, ASI deployed thirty-five (n = 35) 5th generation families (5 consecutive years of biparental breeding) of juvenile *C. gigas* into three areas known to harbour the OsHV-1 virus, the Georges River (New South Wales, Australia; 34.035S, 151.145E), Pipe Clay Lagoon (Tasmania, Australia; 42.970S, 147.525E) and Pittwater (Tasmania, Australia; 42.802S, 147.509E) (Kube et al., 2018). Based on these field disease-exposure studies, expected breeding values (EBVs) were calculated by ASI. These EBVs are an estimation of how well the oysters will perform for a particular trait and the likelihood of passing those traits to their progeny. For the purposes of this study, families were classified into 'resistance groups' (RG) based on their OsHV-1 μvar disease resistance EBV. Families with an EBV greater than 0.6 were placed into RG1 (high disease-resistance), those with an EBV greater than 0.3 and less than 0.6 were placed into RG2 (medium disease-resistance), and families with an EBV less than 0.3 were placed into RG3

(low disease-resistance) (Table 3.1). The estimated heritability is the likelihood of the offspring demonstrating a particular trait, in this case OsHV-1 μvar disease resistance. Resistance is determined by the combination of many genes, since the stock used are derived from a number of genetically distinct families, each family differs in its resistance, and crosses between families differ.

In addition to disease-resistance, EBVs of other oyster traits were also provided by ASI. These traits include: meat condition, the ratio of wet meat to the total weight; depth index, the ratio of shell depth to shell length; shell length; oyster weight, including the oyster shell; and width index, the ratio of shell width to shell length. As EBV's are proprietary information, rather than providing absolute values for each index, we generated a 'rank' system to categorise families according to each index, with ranks of 1 being the highest (Table 3.1).

Table 3.1: Expected breeding value ranks for the studied oyster families including OsHV-1 µvar disease-resistance.

Family line	OsHV-1 µvar resistance	Resistance group (RG)	Meat condition	Depth index	Shell length	Oyster weight	Width index
F_01	8	RG2	22	6	28	7	7
F_02	25	RG3	20	1	29	10	10
F_03	6	RG2	17	18	21	13	13
F_07	16	RG2	6	4	34	1	1
F_10	26	RG3	16	10	32	5	5
F_11	24	RG3	11	7	31	6	6
F_15	29	RG3	6	4	34	1	1
F_16	31	RG3	3	14	11	23	23
F_19	17	RG2	17	18	21	13	13
F_20	28	RG3	4	20	9	13	13
F_23	15	RG2	13	28	19	19	19
F_25	20	RG2	6	21	19	20	20
F_26	32	RG3	12	8	25	22	22
F_27	22	RG3	25	2	27	17	17
F_29	7	RG2	27	31	2	35	35
F_30	18	RG2	23	30	3	33	33
F_35	34	RG3	1	27	10	8	8
F_36	10	RG2	9	24	17	24	24
F_37	12	RG2	4	9	30	11	11
F_39	27	RG3	2	15	17	9	9
F_40	11	RG2	17	12	26	21	21
F_43	19	RG2	20	10	21	28	28
F_51	23	RG3	14	28	13	12	12
F_61	30	RG3	14	35	1	34	34
F_62	33	RG3	24	24	7	17	17
F_65	13	RG2	30	31	3	30	30
F_66	1	RG1	30	31	3	30	30
F_67	9	RG2	30	31	3	30	30
F_68	3	RG1	30	16	15	25	25
F_69	5	RG1	30	16	15	25	25
F_72	2	RG1	35	21	13	16	16
F_77	4	RG1	26	24	8	25	25
F_80	21	RG3	29	23	12	29	29
F_84	14	RG2	28	2	33	3	3
F_86	35	RG3	10	13	24	4	4

Survival data was found after deployment in three different OsHV-1 µvar positive estuaries across Australia. Expected breeding values (EBVs), including OsHV-1 µvar disease-resistance, are shown as a rank number out of 35.

For this microbiome study, the families were deployed into the Georges River (34.035S, 151.145E) on the 16th of August 2016 and sampled two months after deployment date. The two-month deployment time was the first opportunity to sample the deployed juvenile oysters and was sufficient time to ensure no evidence of disease or morbidity. Oysters were deployed in a resolvable incomplete block design to account for micro-geographic variation, blocks were subsections of a replicate and there were three replicates for each family, with each family stocked into a subsection of the tray (Kube et al., 2018). Five oysters from each of the 35 families (total = 175 samples) were sampled and immediately placed on ice and transported to the laboratory where they were stored at -80 °C until further processing.

3.3.2 DNA extraction, sequencing and bioinformatics

The outer shell of the five sampled oysters was rinsed under running tap water to remove any remaining mud and debris. Defrosted oysters were then shucked with sterilised shucking knifes and approximately 25 mg of adductor muscle tissue was aseptically removed using sterile scalpel blades.

The Qiagen DNeasy blood and tissue kit (catalogue: 69506) was used to extract DNA samples, as per the manufacturer's instructions. Microbial community composition within samples was subsequently assessed using 16S rRNA amplicon sequencing, whereby the ribosomal 16S rRNA V1-V3 region was targeted using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') primer pair. The PCR cycling conditions were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplicons were sequenced using the Illumina MiSeq platform (2 x 300 bp) using standard approaches (Ramaciotti Centre for Genomics at the University of New South Wales, Sydney, Australia). Raw data files in

FASTQ format were deposited in the NCBI Sequence Read Archive (SRA) under the Bioproject number PRJNA497763.

Briefly, 16S rRNA paired-end DNA sequences were joined using Flash (Magoc and Salzberg, 2011) and subsequently trimmed using Mothur (Schloss et al., 2009b) (Parameters: maxhomop=5, maxambig=0, minlength=432, maxlength=506). The resulting fragments were clustered into operational taxonomic units (OTUs) at 97% sequence identity, and chimeric sequences were identified using vsearch (Rognes et al., 2016). Taxonomy was assigned in QIIME (Caporaso et al., 2010) using the uclust algorithm (Edgar, 2010) against the Silva v128 database. Mitochondrial and chloroplast data were filtered out of the dataset and the remaining data were rarefied to allow for even coverage across all samples (Supplementary Datasheet 2). OTUs representing less than 0.1% relative abundance in an individual sample were also filtered from the dataset (Supplementary Table 3.1 and Supplementary Table 3.2).

3.3.3 Core microbiome analysis

To determine whether a core oyster microbiome could be characterised, we examined the microbiome of oysters at three different thresholds. First, for individual families, then for RGs, then for all samples together. A core OTU was defined as an OTU that was present in at least all but one replicate (to account for outliers) within a family. To achieve this, the panbiom.py script was used as detailed in (Kahlke, 2017). Briefly, the final biom file generated during the QIIME analysis was used in conjunction with a treatment file that identifies which samples are replicates within a family. The panbiom.py arguments were as follows: a replicate threshold of 1 (-r parameter) and an outlier threshold of ‘x’ (-x parameter). The –x parameter treats the replicate

threshold value as an outlier threshold value, simply put, it can be absent in one replicate sample (indicated by –r = 1 and –x = x).

3.3.4 Quantitative PCR (qPCR)

Due to the potential role of *Vibrio* in OsHV-1 µvar disease dynamics (Segarra et al., 2010; Jenkins et al., 2013; Lemire et al., 2015; Petton et al., 2015; De Lorgeril et al., 2018), quantitative PCR (qPCR) was used to examine patterns in *Vibrio* abundance across the RGs. qPCR was performed using an epMotion 5075l Automated Liquid Handling System on a Bio-Rad CFX384 Touch Real-Time PCR Detection System with a six-point calibration curve and negative controls on every plate. The calibration curve was built from a known amount of amplicon DNA measured by Qubit, followed by a ten-fold dilution to fill out the calibration curve. All sample analyses were performed with three technical replicates, using the following reaction mixture: 2.5 µL iTaq Universal SYBR Green supermix, 0.4 µM of each forward and reverse primer, 1 µL of diluted (1:15) template DNA, and the remainder made up with water. To quantify abundance of the *Vibrio* community, the *Vibrio*-specific 16S rRNA primers Vib1-f (5'-GGCGTAAAGCGCATGCAGGT -3') and Vib2-r (5'-GAAATTCTACCCCCCTCTACAG -3') were used (Thompson et al., 2004; Vezzulli et al., 2011; Siboni et al., 2016). The qPCR cycling conditions were as follows: 95 °C for 3 minutes followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The resulting data were normalised to both elution volume (200 µL) and tissue weight. A coefficient of variation (CV) was then calculated for the technical triplicates, and samples with CV > 10% were removed from the analysis. A melting curve was added to the end of every run to confirm the presence of a single PCR product.

3.3.5 Statistical analysis

Comparisons of alpha diversity were performed with a One-Way ANOVA followed by a Tukey's pairwise test. Normalised [square root (x)] data were used to compare community compositions using a non-metric multidimensional scaling analysis (nMDS) with a Bray-Curtis similarity index. To determine significantly different microbial assemblage between families and RGs, and to compare qPCR data, a one-way PERMANOVA was used. To examine which OTUs contributed to differences between RGs, a SIMPER analysis with a Bray-Curtis similarity index was used. To define associations between breeding values and OTUs, breeding values were normalised (x-mean/standard deviation) and used within a canonical correspondence analysis (CCA). All analyses were performed using the PAST statistical software (Hammer et al., 2001). To determine whether an OTU was significantly elevated in a particular RG, the group_significance.py script using the default analysis (Kruskal Wallis ANOVA) was used in QIIME. To examine correlations between EBVs, we performed a maximal information-based nonparametric exploration (MINE) analysis (Reshef et al., 2011).

3.4 Results

3.4.1 The C. gigas microbiome

Following data filtering and rarefication, a total of 3294 OTUs were observed across the entire dataset, and of these, 68.5% occurred at below 1% of the total relative abundance. Conversely, across all samples and spanning all RGs, a member of the *Pseudomonas* genus (OTU 2034) was found to be the most relatively abundant OTU comprising 5.6% of the bacterial community. This was followed by OTUs matching an uncultured bacterium in the *Psychrobacter* genus (OTU 1488) and an uncultured bacterium in the

Mycoplasma genus (OTU 3150), which represented 4.8% and 4% of the *C. gigas* microbiome across the whole dataset respectively (Figure 3.1).

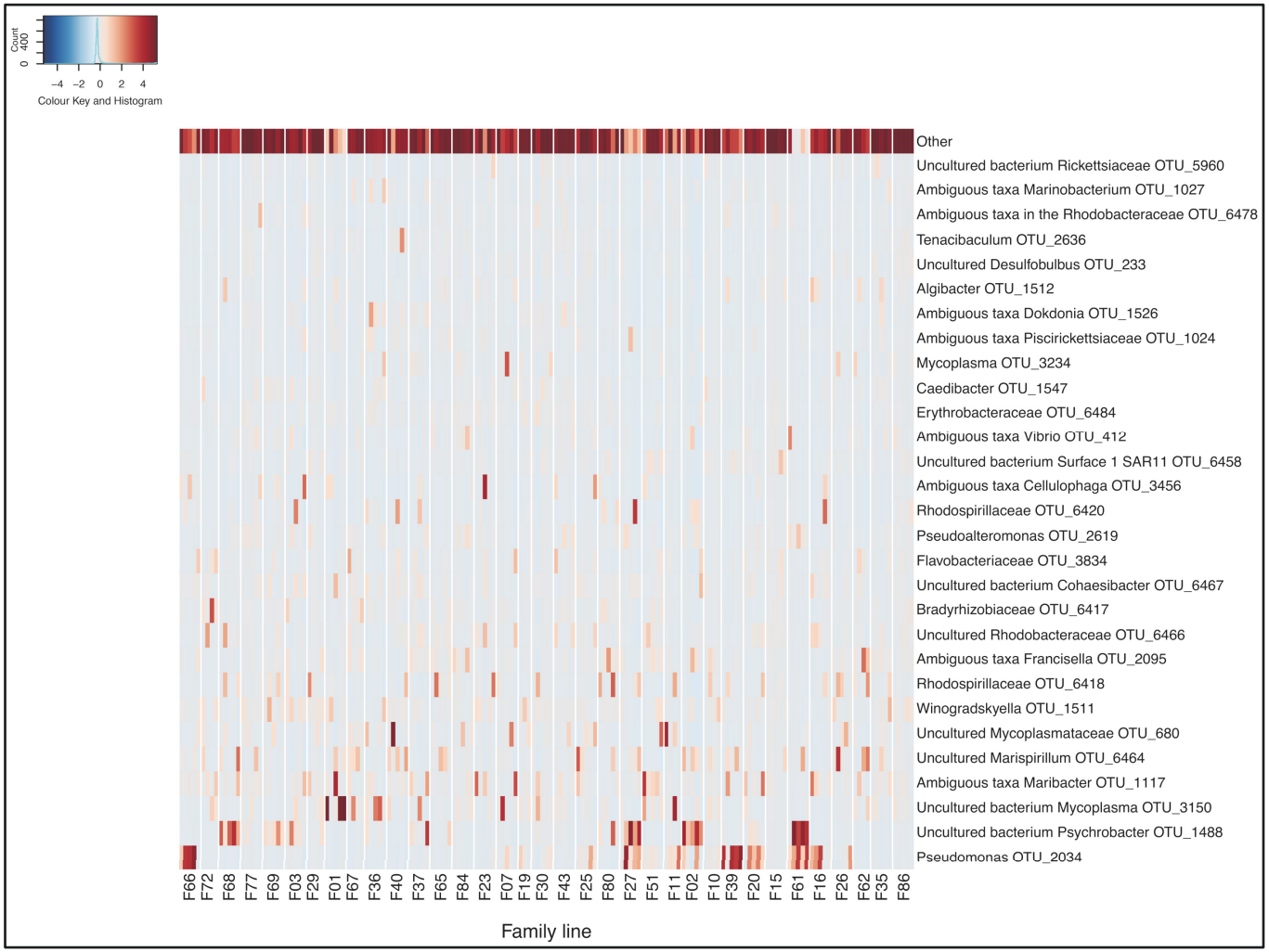


Figure 3.1: Heatmap of scaled OTU relative abundance for the 30 most abundant OTUs as well as the remaining summed lowly abundant OTUs. Families ordered by OsHV-1 µvar disease-resistance. Heatmap was made using the R statistical environment using scaled data with the gplots and RColorBrewer packages (Neuwirth, 2014; Warnes et al., 2016; R_Core_Team, 2017).

3.4.2 *Variability in the C. gigas microbiome across different resistance lines*

To determine whether breeding for disease resistance influences the *C. gigas* microbiome, the microbiome of oysters assigned to RGs were characterised and compared. Alpha diversity, quantified using Shannon's diversity index was significantly higher in RG2 when compared to the RG3 RG ($F_{(1, 141)} = 6.8$, $p = 0.025$), but did not vary significantly when compared to RG1 ($F_{(1, 93)} = 0.4$, $p = 0.51$). Species richness (Chao1) did not differ significantly between any of the RGs (RG1 vs RG2 - $F_{(1, 93)} = 0.03$, $p = 0.85$; RG1 vs RG3 - $F_{(1, 94)} = 1.3$, $p = 0.26$; RG2 vs RG3 - $F_{(1, 141)} = 1.08$, $p = 0.30$).

Comparisons of microbiome composition (beta diversity) across different RGs revealed that the microbiomes of RG1 and RG2 were both significantly different to the least disease resistant group, RG3 ($p = 0.019$ and $p = 0.0001$; $F_{(1, 94)} = 1.47$ and $F_{(1, 141)} = 2.93$ respectively). No significant difference was found between the microbiomes of RG1 and RG2 ($F_{(1, 93)} = 1.29$, $p = 0.055$). Statistical comparisons between RG2 and RG3 appeared to be stronger than those between RG1 and RG3, possibly due to more families being assigned to RG2, therefore potentially adding more microbiome variability to this group. No clear dissimilarity in the microbiome of the RGs was apparent in a 3D nMDS (Stress = 0.34), or a PCoA (Supplementary Figure 3.1). SIMPER comparisons showed that the composition of the microbiomes associated with RG1 and RG2 were 81.83% and 82.12% dissimilar to RG3 respectively (Supplementary Table 3.3; Supplementary Table 3.4).

As the RG with the lowest level of disease-resistance (RG3) was found to have a significantly different microbial assemblage to both RG2 and RG1, we examined which OTUs were responsible for driving the differences in microbiome structure between these groups (Figure 3.2). An OTU assigned to the *Pseudomonas* genus (OTU 2034; the most abundant OTU in the entire

dataset) was over-represented in the RG3 microbiome relative to both RG1 ($H_{(1, 94)} = 7.6$, $p = 0.0058$) and RG2 ($H_{(1, 141)} = 15$, $p = 0.00011$). Conversely, an OTU assigned to the *Tenacibaculum* genus (OTU 2636) and two separate OTUs assigned to the *Dokdonia* genus (OTUs 2162 and 1526) were all significantly under-represented in RG3 (*Tenacibaculum* RG1 $H_{(1, 94)} = 4.5$, $p = 0.033$ and RG2 $H_{(1, 141)} = 15.2$, $p = 0.0056$; *Dokdonia* RG1 $H_{(1, 94)} = 7.7$, $p = 0.0001$ and RG2 $H_{(1, 141)} = 30.3$, $p < 0.0001$).

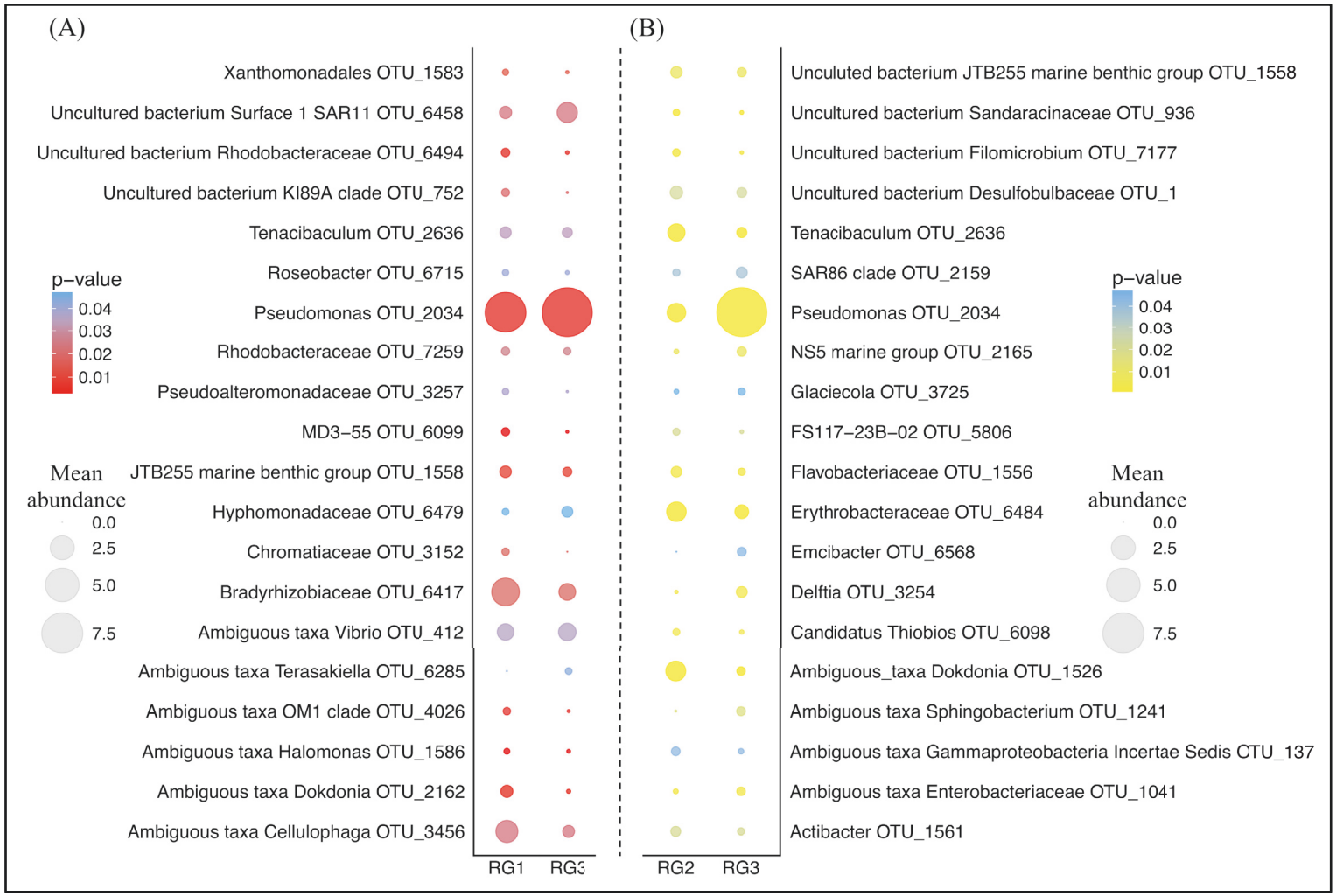


Figure 3.2: Bubble plot of group_significance.py analysis results using the default Kruskal-Wallis parameters. **(A)** represents the comparison between RG1 and RG3. **(B)** represents the comparison between RG2 and RG3. Colour represents the strength of the p-value. Size represents the mean relative abundance of that OTU across the whole resistance group (RG). OTUs assigned to the genus and species level were chosen, and those 20 most abundant from each RG are displayed.

Notably, a member of the *Vibrio* genus (OTU 412) was found to be significantly over-represented in the least disease-resistant group (RG3) relative to the most disease-resistant group (RG1) ($H_{(1, 94)} = 4.4$, $p = 0.036$). Due to the previously demonstrated importance of *Vibrio* species in OsHV-1 µvar infection (Segarra et al., 2010; Jenkins et al., 2013; De Lorgeril et al., 2018), we subsequently employed a *Vibrio*-specific 16S rRNA qPCR assay to compare total abundances of *Vibrio* across RGs. A significant elevation of *Vibrio* 16S rRNA gene copies was observed in RG3 compared to RG1 ($F_{(1, 94)} = 2.86$, $p = 0.027$) and RG2 ($F_{(1, 141)} = 3.25$, $p = 0.014$) (average of 179, 107 and 75 gene copies mg of tissue^{-1} respectively; Supplementary Figure 3.2). Furthermore, OTUs assigned to the *Vibrio* genera were significantly elevated in RG3 when compared to RG1 ($F_{(1, 94)} = 4.27$, $p = 0.011$), but not RG2 ($F_{(1, 141)} = 2.48$, $p = 0.07$). To determine the extent of whether *Vibrio* OTUs were driving the differences between RG1 and RG3 microbiomes, OTUs assigned to the *Vibrio* genus were removed and the RG beta diversity comparison was reperformed. When doing this, we observed a slight weakening of the statistical comparison between RG1 and RG3, from ($F_{(1, 94)} = 1.47$, $p = 0.019$) to ($F_{(1, 94)} = 1.46$, $p = 0.024$).

A CCA was used to highlight associations between specific OTUs, OsHV-1 µvar disease-resistance and EBVs of other traits (Figure 3.3). OTUs matching the *Cupriavidus* (OTU 2182) and *Psychrilyobacter* (OTU 5046) genera were closely coupled with disease-resistance, followed by a member of the *Tenacibaculum* (OTU 2153) genus and an uncultured bacterium in the *Frankiales* order (OTU 5180). While OTUs assigned to members of the *Photobacterium* (OTU 1063; OTU 654; OTU 1053), *Vibrio* (OTU 651; OTU 653) and *Aliivibrio* (OTU 1248) genera were negatively associated with disease-resistance, but strongly associated with meat condition. Furthermore, members of the *Streptococcus* (OTU 814) and *Roseovarius*

(OTU 7180) genera were closely associated with depth and width index, and also negatively associated with disease-resistance. The community composition was largely influenced by the first axis, driven by growth related EBVs. A MINE analysis identified a negative correlation between disease resistance and width index ($p = 0.047$; linear regression = -0.34), and a positive correlation between disease resistance and oyster weight ($p = 0.038$; linear regression = 0.15). Shell length and depth index had the strongest negative correlation ($p = <0.001$; linear regression = -0.92), while oyster weight and shell length had the strongest positive correlation ($p = 0.002$; linear regression = 0.74; Supplementary Table 3.5).

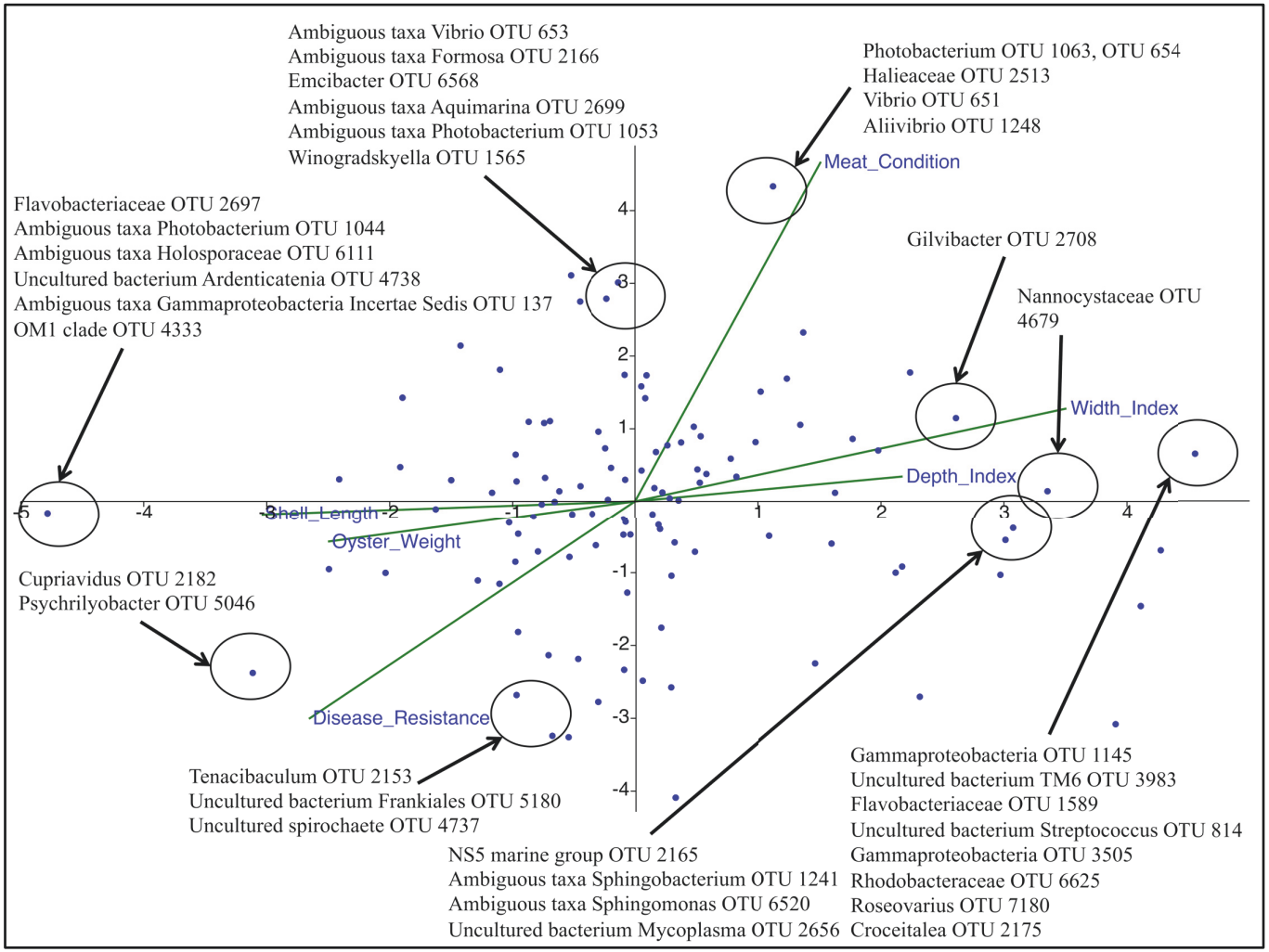


Figure 3.3: Canonical correspondence analysis plot using 3% relative abundance filtered data. *Cupriavidus*, *Psychrilyobacter*, *Tenacibaculum* and *Frankiales* were found to be strongly associated with OsHV-1 µvar disease-resistance, while OTUs assigned to the *Photobacterium*, *Vibrio* and *Aliivibrio* were negatively associated with OsHV-1 µvar disease-resistance. Axis 1 and 2 were able to significantly represent 53.2% of the data ($p = 0.001$ for both axes with 999 permutations).

3.4.3 Defining the core C. gigas microbiome across different resistance lines

Due to the dynamic nature of oyster microbiomes, identifying a core microbiome can provide insights into which members may be driving the within-microbiome interactions and possibly shaping the community composition. While we were unable to identify a universal core microbiome across all samples, analyses of individual families revealed that each family had a small core microbiome (9-109 OTUs), with many of these OTUs shared across families. Families 30 and 84, within RG2, shared the most core OTUs (4) (Supplementary Figure 3.3). In contrast, family 19 of RG2 had the most unique core OTUs (27), that is those core OTUs not shared with any other family. To determine how many unique core OTUs were present in each oyster family (and therefore each RG), we compiled all of the core OTUs from the core analysis and removed duplicate bacteria. When doing this, a total of 9, 54 and 16 unique OTUs were assigned to RG1, RG2 and RG3 respectively (Table 3.2). When performing a separate core analysis on each RG as a whole, RG1 was comprised of two core members, a member of the *Winogradskyella* genus (OTU 1511) and a member of the *Bradyrhizobiaceae* family (OTU 6417). While, no core bacterial members were found for RG2 or RG3 microbiomes.

Table 3.2: Unique core bacterial members from individual oyster families collated into their respective resistance groups (RG).

Resistance group	Combined unique core members from individual family lines	
RG1	Acinetobacter OTU_2667	Rhodobacteraceae OTU_6650
	Ambiguous taxa Cellulophaga OTU_3456	Ambiguous taxa Marinomonas OTU_1295
	Brevundimonas OTU_6676	Roseobacter OTU_6715
	Ambiguous taxa Marinomonas OTU_1295	Rhodobacteraceae OTU_7212
	Ambiguous taxa Ilumatobacter OTU_4817	
RG2	Uncultured Salinimonas OTU_6618	Sva0725 OTU_433
	Planctomycetaceae OTU_4123	Uncultured bacterium OM1 clade OTU_4332
	Ambiguous taxa Gammaproteobacteria Incertae Sedis OTU_465	Uncultured bacterium Sva0996 marine group OTU_4477
	Uncultured bacterium Anaerolineaceae OTU_4681	Ambiguous taxa Ilumatobacter OTU_4840
	Uncultured bacteria Gammaproteobacteria OTU_5902	Ambiguous taxa Sva0996 marine group OTU_4982
	Croceitalea OTU_2175	Uncultured bacterium Ardenticatenia OTU_5150
	Uncultured bacterium Halanaerobiales ODP1230B8.23 OTU_4816	Rhodobiaceae OTU_6148
	Oceanospirillaceae OTU_1577	Rhodobacteraceae OTU_6485
	Pseudoalteromonadaceae OTU_3257	Sphingomonadales OTU_6620
	Uncultured bacterium Ilumatobacter OTU_4687	Marivita OTU_6626
	Uncultured bacterium Acidobacteria Subgroup 21 OTU_5711	Beijerinckiaceae OTU_6687
	Rhodobacteraceae OTU_6481	PAUC43f marine benthic group OTU_699
	Ambiguous taxa Acidobacteria Subgroup 9 OTU_7	Anderseniella OTU_7187
	Persicirhabdus OTU_866	Ambiguous taxa Sandaracinaceae OTU_1154
	Uncultured bacterium Ralstonia OTU_1255	Ambiguous taxa Thiogranum OTU_1467
	Flavobacteriaceae OTU_1551	Ambiguous taxa Holophagae Subgroup 23 OTU_251
	Ambiguous taxa Profundimonas OTU_1559	Candidatus Thiobios OTU_6098
	Ambiguous taxa JTB255 marine benthic group OTU_1588	JTB255 marine benthic group OTU_1566
	JTB255 marine benthic group OTU_1642	OM190 OTU_2018
	Gilvibacter OTU_2167	
	Myxococcales OTU_238	

	Ambiguous taxa Flavobacteriaceae OTU_3100	Uncultured bacterium Holophagae Subgroup 23 OTU_240
	Uncultured bacterium Pir4 lineage OTU_3294	Uncultured bacterium Belgica2005-10-ZG-3 OTU_25
	Halieaceae OTU_3519	Ambiguous taxa Roseibacillus OTU_2529
	Uncultured bacterium OM1 clade OTU_4096	OM60(NOR5) clade OTU_3504
	Planctomycetaceae OTU_4100	Uncultured bacterium Desulfobulbus OTU_404
	Ambiguous taxa OM1 clade OTU_4271	Ambiguous taxa Sva0996 marine group OTU_4976
	Planctomycetaceae OTU_4278	Ambiguous taxa Acidobacteria Subgroup 17 OTU_785
RG3	Rhizobiales OTU_6486	
	Uncultured bacterium Rickettsiaceae OTU_5903	Desulfobulbus OTU_235
	Vibrionaceae OTU_655	Uncultured bacterium Emcibacter OTU_6286
	Ambiguous taxa Sphingobacterium OTU_1241	Rhodobacteraceae OTU_7097
	Ambiguous taxa NS4 marine group OTU_2698	Mycoplasma OTU_3722
	Pseudomonas OTU_3032	Uncultured bacterium Phyllobacteriaceae OTU_6619
	Gammaproteobacteria OTU_3505	Ruegeria OTU_6653
	Roseovarius OTU_7180	Uncultured bacterium Rhodobacteraceae OTU_7173
	Uncultured bacterium Maribacter OTU_1486	

Each family was found to have a core microbial community, the displayed core OTUs are those not shared with any other family line. RG1 is the most disease RG, RG2 is an intermediate RG, and RG3 is the most disease susceptible group.

3.5 Discussion

The principal goal of this study was to identify patterns in the *C. gigas* microbiome across 35 oyster families with differing levels of resistance to OsHV-1 µvar disease, with the objective of elucidating microbial taxa associated with disease resistance. Immunosuppression from OsHV-1 µvar infection allows opportunistic bacteria within the oyster's microbiome to induce bacteraemia, killing the host (Petton et al., 2015; De Lorgeril et al., 2018). Characterising these interactions and gaining insights into the oyster microbiome is essential to further understand the dynamic interplay between the microbiome, OsHV-1 µvar and disease. A significant difference in the structure of the microbiome of oysters exhibiting different levels of resistance to OsHV-1 µvar disease was observed. Specifically, the microbiomes associated with the oysters showing the most resistance to OsHV-1 µvar disease (RG1) and moderately resistant oysters (RG2) were significantly different to the most disease susceptible (or least resistant) group (RG3). When considering disease resistance, we observed a strong negative association between the OsHV-1 µvar disease resistance of oyster hosts and the occurrence of OTUs assigned to the *Vibrio* (OTUs 651 and 653), *Photobacterium* (OTUs 1063, 654 and 1053), *Aliivibrio* (OTU 1248), *Streptococcus* (OTU 814) and *Roseovarius* (OTU 7180) genera, while on the other hand, the microbiomes of the most resistant families had an over-representation of OTUs assigned to the *Cupriavidus* (OTU 2182), *Psychrilyobacter* (OTU 5046) and *Tenacibaculum* (OTU 2153) genera.

The association between the occurrence of *Vibrio* and disease susceptibility was further supported by a significant elevation of an uncharacterised member of the *Vibrio* in RG3, and the results of a *Vibrio*-specific qPCR assay. These results are consistent with growing evidence implicating a role

of the *Vibrio* community in oyster disease (Sugumar et al., 1998; Waechter et al., 2002; Garnier et al., 2007; Saulnier et al., 2010; Lemire et al., 2015; Petton et al., 2015; Green et al., 2019; King et al., 2019a). Specifically, there is previous evidence that prior to oyster disease onset, the native *Vibrio* community is replaced by pathogenic *Vibrio* species (Lemire et al., 2015). Further, in corals, small shifts in the *Vibrio* community are sufficient to shift the microbiome metabolism (Thurber et al., 2009). Our data provides a new perspective on this interaction, whereby the total load of *Vibrios* differed between disease susceptible and resistant oyster families. This is supported by a recent study, which demonstrated that the *Vibrio* load following OsHV-1 μvar infection was significantly higher in disease-susceptible oysters (De Lorgeril et al., 2018). An increased *Vibrio* community size may provide further potential for pathogenic species to replace benign colonisers. On the other hand, a higher background load of *Vibrio* may become important under periods of stress, such as with OsHV-1 μvar infection, resulting in duel infection, as has recently been described (De Lorgeril et al., 2018). This is also indirectly supported by a previous study which observed reduced mortality in OsHV-1 infected oysters that were treated with antibiotics (Petton et al., 2015).

Increases in the abundance of OTUs assigned to the *Photobacterium* genus, as were observed here, often co-occur with an increase in the *Vibrio* community in oyster microbiomes (Wegner et al., 2013; Lokmer and Wegner, 2015). While members assigned to this genus have been identified as pathogens of other aquatic organisms (Pedersen et al., 2009; Liu et al., 2016), to our knowledge, no species of *Photobacterium* has been identified as an oyster pathogen. Members of the *Streptococcus* and *Aliivibrio* genera are known pathogens of fish and crabs (Pappalardo and Boemare, 1982; Egidius et al., 1986; Creeper and Buller, 2006; Urbanczyk et al., 2007), while

a member of the *Roseovarius* genus is the causative agent of roseovarius oyster disease (formally juvenile oyster disease) in *Crassostrea virginica* (Boettcher et al., 2005; Maloy et al., 2007), yet to our knowledge these genera have not been implicated in disease of *C. gigas* previously, despite being over-represented in the most disease susceptible oyster families.

On the other hand, a strong positive association was observed between levels of disease resistance and the occurrence of OTUs assigned to the *Cupriavidus* (OTU 2182), *Psychrilyobacter* (OTU 5046) and *Tenacibaculum* (OTU 2153). Currently, little is known about the role of these genera in oysters. *Cupriavidus* species are commonly isolated from plants and soil (Cuadrado et al., 2010; Estrada-De Los Santos et al., 2014), but members of the *Psychrilyobacter* and *Tenacibaculum* have previously been observed in *C. gigas* microbiomes (Lee et al., 2009; Fernandez-Piquer et al., 2012; Wegner et al., 2013). *Psychrilyobacter* was observed in *C. gigas* microbiomes from Tasmania, Australia (Fernandez-Piquer et al., 2012), which is perhaps notable given that the oysters used in this study were initially sourced from Tasmania. In addition, we have previously identified an over-representation of a *Tenacibaculum* OTU in oyster microbiomes that were unaffected by a summer mortality outbreak (King et al., 2019a).

As already stated, a significant elevation of OTUs belonging to the *Vibrio* and *Photobacterium* genera abundance in disease susceptible oysters has also been previously observed (De Lorgeril et al., 2018), supporting our findings. However, while we identified members of the *Psychrilyobacter* and *Tenacibaculum* genera to be associated with disease resistance, the same study (De Lorgeril et al., 2018) observed an increase in these same genera in an experimental infection experiment using disease susceptible oysters. Differences in bacterial taxa abundance and taxonomic assignment could be

attributed to contrasting sequencing techniques and data analysis. For example, we used the V1-V3 hypervariable region, and clustered OTUs at the 97% identity level, compared to V3-V4 and having OTUs clustered at a 3-nucleotide difference threshold (De Lorgeril et al., 2018). Furthermore, this study deployed oysters to the field, while the aforementioned study carried out their experiments in tanks. Tank based studies are known to significantly alter the oyster microbiome composition compared to oysters sourced from the environment (Lokmer et al., 2016a).

The oyster microbiome is dynamic in nature, changing in response to stressors such as disease, antibiotics, translocation, and heat (Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016b; De Lorgeril et al., 2018; Green et al., 2019; King et al., 2019a). The microbiome assemblage can also be influenced by the oyster life stage, the genetics of the host oyster, and spatial location (Trabal et al., 2012; Wegner et al., 2013; Lokmer et al., 2016a; King et al., 2019a). Because we only have one sampling point, our study would not capture the dynamic nature of the oyster microbiome, and thus the oyster microbiome could change before the onset of disease. To fully capture the importance of the taxa identified in this study, a temporal study in the field encompassing a disease outbreak would be needed. However, as disease outbreaks are often very sudden, capturing a disease outbreak in the environment can be difficult.

In addition to identifying OTUs that are over- or under-represented within the microbiomes of oysters with different levels of disease-resistance, another way to identify putatively important bacteria within the microbiome of a host organism involves the identification of “core” microbiome members (Ainsworth et al., 2015). Identifying which bacterial members are consistent and stable across microbial communities is important in

unravelling the functional contribution of these core bacteria (Ainsworth et al., 2015). Notably, we could not define a universal core microbiome across all of the studied oyster families at the OTU level, suggesting significant heterogeneity in oyster microbiome structure, or possible differences in micro-geographic variation. However, we identified core microbiome members within each family microbiome, whereby a number of 'unique' core members often occurred exclusively in the core microbiome of a family. This is in accordance with previous observations that the composition of an oyster's microbiome is partially governed by oyster genetics, particularly for shaping the rare specialist bacterial community (<1% abundance) (Wegner et al., 2013), although we have no information pertaining to the genetic differentiation between the studied oyster families. However, when examining the core microbiome across all of the families comprising the most highly disease-resistant group (RG1), we identified two core members, which included OTUs classified as members of the *Winogradskyella* genus (OTU 1511) and *Bradyrhizobiaceae* family (OTU 6417). OTUs assigned to the *Bradyrhizobiaceae* family have previously been observed in oysters (Sakowski, 2015), however, due to the coarse taxonomic assignment of this OTU, it is unclear what potential role this member of the *Bradyrhizobiaceae* family might have. *Winogradskyella* species are commonly found in numerous marine organisms, including oysters (Valdenegro-Vega et al., 2013; Park et al., 2015; Lee et al., 2017; Schellenberg et al., 2017; Franco et al., 2018), and are known for their role in amoebic-induced fish gill diseases (Embar-Gopinath et al., 2005; Embar-Gopinath et al., 2006). However, it is uncertain what function(s) *Winogradskyella* species play in oysters. We currently know little about the potential role, if any, of these core microbiome members in resistance, but these observations provide candidate target organisms for focussed examinations of potential beneficial microbes within OsHV-1 μvar disease-resistance.

3.6 Conclusion

We have shown that the microbiome of *C. gigas* displays significantly different microbial assemblage structure according to oyster disease-resistance. This study provides insights into the *C. gigas* microbiome within the context of oysters bred for disease-resistance and highlights the potential involvement of the oyster microbiome in disease-resistance. Members of the *Vibrio*, *Photobacterium*, *Aliivibrio*, *Streptococcus* and *Roseovarius* genera were over-represented features of the microbiome of oysters with high OsHV-1 μvar disease susceptibility, which is consistent with previous studies implicating *Vibrio* in oyster disease dynamics. Furthermore, a significant elevation of *Vibrio* 16S rRNA gene copies in disease-susceptible oyster families could indicate a lack of immune response against *Vibrio* pathogens. However, further research is required to elucidate the role of these bacteria in oyster disease dynamics. Examination of 'core' bacteria identified species assigned to the *Winogradskyella* genus and *Bradyrhizobiaceae* family as core members of microbiomes assigned to RG1 and may also play a role in OsHV-1 μvar disease resistance. These results deliver evidence that the *C. gigas* microbiome differs between oysters with different levels of susceptibility to OsHV-1 μvar disease and identifies putative microbial determinants in disease onset and resistance.

3.7 Data availability

The datasets generated for this study can be found in NCBI SRA, PRJNA497763.

3.8 Author contributions

WK and KN carried out the fieldwork. WK and NW processed the samples. WK and NS analysed the data. CJ, MD, WO'C, JS, and ML conceived and

designed the study. TK produced the core microbiome analysis. WK, JS, and ML wrote the manuscript.

3.9 Funding

This research was supported by an Australian Research Council Linkage Project (LP160101795) to JRS and ML; 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.

3.10 Supplementary Information

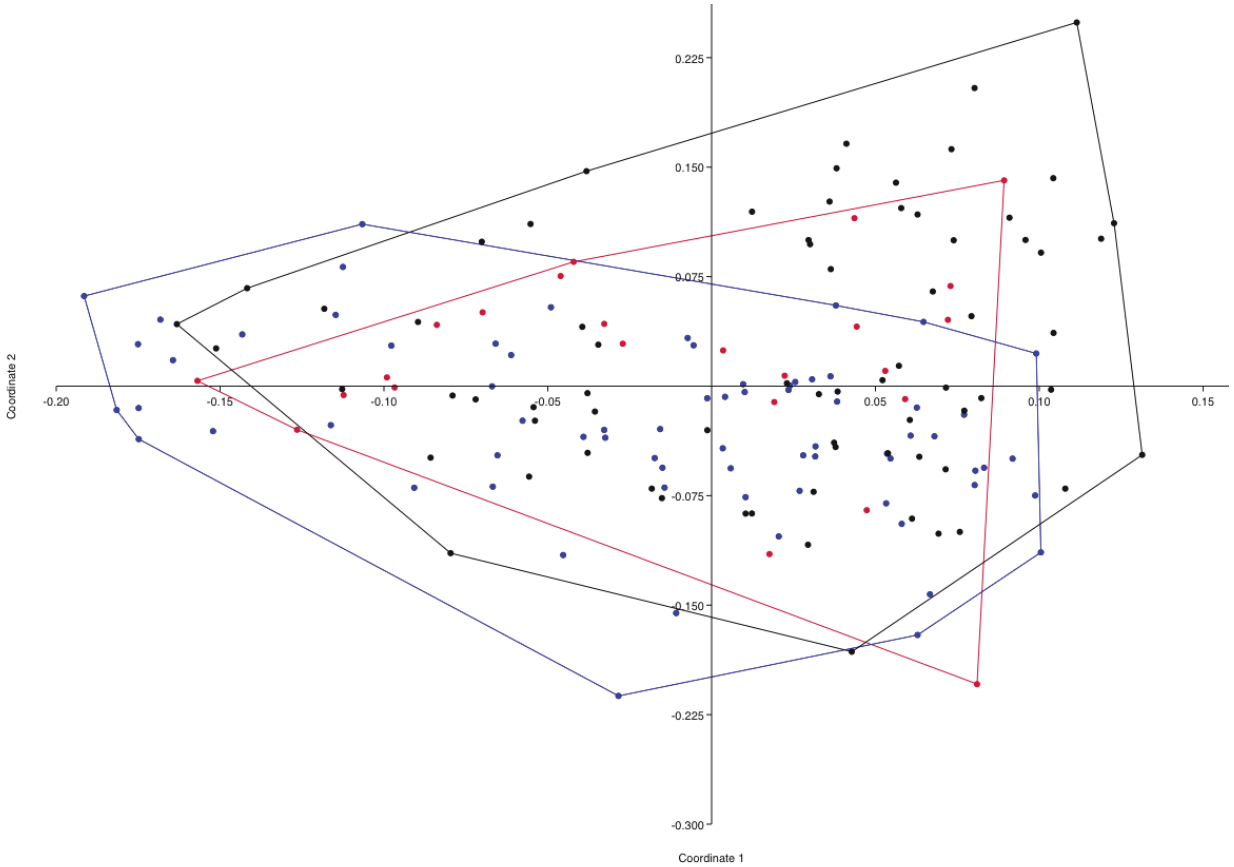
Supplementary Table 3.1 Information pertaining to the data analysis, including the number of reads before and after cleaning, and rarefaction at 6000 reads per sample.

Information	Read/Observation number
Reads after trimming	4847965
Reads after cleaning	3961808
Observations (OTUs) after cleaning	4188
Samples before rarefaction	175
Reads after rarefaction	1002000
Observations (OTUs) after rarefaction	4188
Reads after filtering below 0.1% prevalence	984544
Observations (OTUs) after 0.1% filtration	3294
Samples after rarefaction	167

Supplementary Table 3.2 Number of reads per sample before rarefication

F29_05:	F30_01:	F43_04:	F77_03:	F84_01:	F02_02:
731.0	10205.0	15877.0	20678.0	24949.0	36633.0
F72_05:	F86_05:	F51_03:	F36_05:	F15_01:	F40_03:
1880.0	10215.0	15919.0	20746.0	25025.0	37209.0
F10_01:	F80_05:	F26_05:	F66_02:	F07_01:	F20_04:
2033.0	10223.0	15990.0	20872.0	25612.0	37217.0
F67_05:	F43_03:	F35_02:	F11_03:	F20_01:	F39_04:
2061.0	10302.0	16359.0	21110.0	25728.0	37323.0
F19_01:	F07_02:	F23_03:	F36_02:	F25_01:	F16_02:
4128.0	10462.0	16468.0	21131.0	25940.0	37486.0
F19_03:	F15_05:	F11_05:	F69_01:	F29_02:	F40_05:
4587.0	10583.0	16469.0	21272.0	26149.0	37565.0
F62_01:	F35_01:	F62_05:	F07_03:	F72_01:	F03_03:
4597.0	10795.0	16607.0	21634.0	26169.0	37590.0
F11_02:	F37_04:	F37_01:	F51_02:	F27_05:	F161_03:
5063.0	11090.0	17028.0	21682.0	26187.0	37681.0
F16_04:	F86_04:	F68_05:	F23_04:	F03_04:	F161_05:
6230.0	11283.0	17041.0	21743.0	26265.0	37814.0
F23_05:	F43_05:	F02_03:	F66_05:	F43_01:	F20_03:
6568.0	11385.0	17135.0	21827.0	26583.0	39270.0
F16_05:	F66_01:	F07_04:	F02_04:	F69_03:	F02_01:
6577.0	11654.0	17364.0	21901.0	26794.0	39548.0
F67_04:	F86_03:	F02_05:	F25_02:	F16_01:	F161_02:
6993.0	11756.0	17365.0	21910.0	27004.0	41347.0
F10_03:	F15_03:	F65_02:	F62_03:	F26_01:	F39_05:
7114.0	12194.0	17554.0	22024.0	27084.0	41547.0
F65_04:	F80_04:	F69_04:	F25_04:	F37_05:	F36_01:
7295.0	12287.0	17596.0	22134.0	27097.0	43010.0
F27_01:	F03_05:	F35_03:	F27_03:	F161_01:	F161_04:
7373.0	12362.0	17972.0	22176.0	28055.0	43782.0
F29_01:	F68_04:	F15_02:	F84_03:	F16_03:	F01_02:
7538.0	12491.0	17985.0	22216.0	28125.0	46130.0
F86_01:	F77_01:	F30_05:	F20_05:	F77_05:	F36_04:
7590.0	12839.0	18074.0	22284.0	28252.0	46154.0

F30_03:	F26_03:	F68_01:	F77_04:	F26_02:	F03_01:
7702.0	12946.0	18220.0	22291.0	28352.0	46315.0
F15_04:	F19_04:	F11_04:	F39_03:	F66_04:	F40_01:
7736.0	13207.0	18400.0	22562.0	28784.0	46664.0
F10_02:	F23_02:	F84_05:	F10_05:	F39_01:	F43_02:
7774.0	13661.0	18593.0	22582.0	29096.0	46703.0
F19_05:	F72_04:	F36_03:	F86_02:	F51_01:	F27_04:
8045.0	14037.0	18782.0	22626.0	29221.0	47122.0
F62_02:	F07_05:	F65_03:	F62_04:	F72_03:	F01_03:
8424.0	14103.0	19453.0	22643.0	29503.0	48356.0
F29_04:	F23_01:	F69_02:	F25_03:	F35_05:	F01_05:
8540.0	14296.0	19735.0	22824.0	30062.0	51053.0
F37_02:	F67_03:	F84_02:	F80_01:	F72_02:	F29_03:
8847.0	14395.0	19880.0	22928.0	30859.0	52418.0
F65_05:	F30_04:	F77_02:	F68_03:	F26_04:	F40_04:
9150.0	14551.0	19980.0	23996.0	30959.0	55924.0
F65_01:	F80_02:	F69_05:	F66_03:	F11_01:	F03_02:
9311.0	14566.0	20120.0	24114.0	31146.0	57403.0
F10_04:	F80_03:	F51_04:	F68_02:	F35_04:	F27_02:
9389.0	15462.0	20441.0	24349.0	32978.0	58607.0
F37_03:	F51_05:	F67_01:	F19_02:	F39_02:	F40_02:
9452.0	15730.0	20468.0	24435.0	33203.0	69726.0
F67_02:	F84_04:	F25_05:	F20_02:	F30_02:	F01_04:
9781.0	15775.0	20541.0	24597.0	35308.0	77185.0
					F01_01:
					78470.0



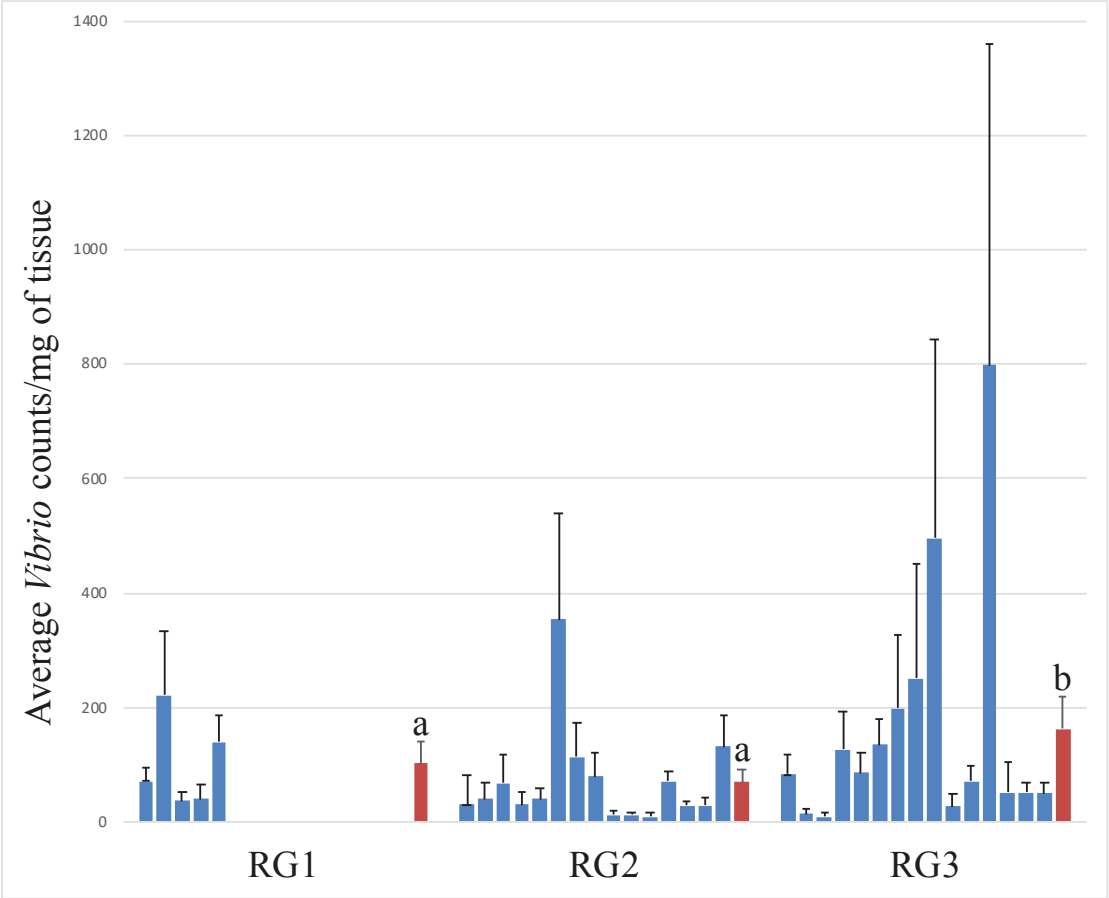
Supplementary Figure 3.1 Principal Coordinates Analysis (PCoA) of oyster microbiomes with a Bray-Curtis similarity index. Blue dots are resistance group 1 (RG1) microbiomes, red dots are resistance group 2 (RG2) microbiomes, and black dots are resistance group 3 (RG3) microbiomes. Axes 1 and 2 represent 10.7% and 6.4% of the data respectively. Transformation exponent c = 6.

Supplementary Table 3.3 SIMPER analysis of resistance group 1 (RG1) microbiomes compared to resistance group 3 (RG3) microbiomes. The top 10 OTUs are displayed with their dissimilarity contribution and transformed mean representation. Dissimilarity contribution is cumulative.

OTU	Dissimilarity (%)	RG1 mean	RG3 mean
Pseudomonas 2034	2.204	1.02	2.05
Uncultured bacterium *Psychrobacter* 1488	2.001	1.81	1.35
Uncultured bacterium *Marispirillum* 6464	1.267	1.03	1.13
Ambiguous taxa *Maribacter* 1117	1.16	1.28	1.29
Rhodospirillaceae 6418	1.062	0.709	0.926
Winogradskyella 1511	0.9522	1.42	1.1
Uncultured bacterium *Mycoplasma* 3150	0.9466	0.598	0.891
Uncultured bacterium *Rhodobacteraceae* 6466	0.9147	0.977	0.903
Uncultured bacterium *Mycoplasmataceae* 680	0.8714	0.759	0.68
Ambiguous taxa *Francisella* 2095	0.8521	0.766	1.14

Supplementary Table 3.4 SIMPER analysis of resistance group 2 (RG2) microbiomes compared to resistance group 3 (RG3) microbiomes. The top 10 OTUs are displayed with their dissimilarity contribution and transformed mean representation. Dissimilarity contribution is cumulative.

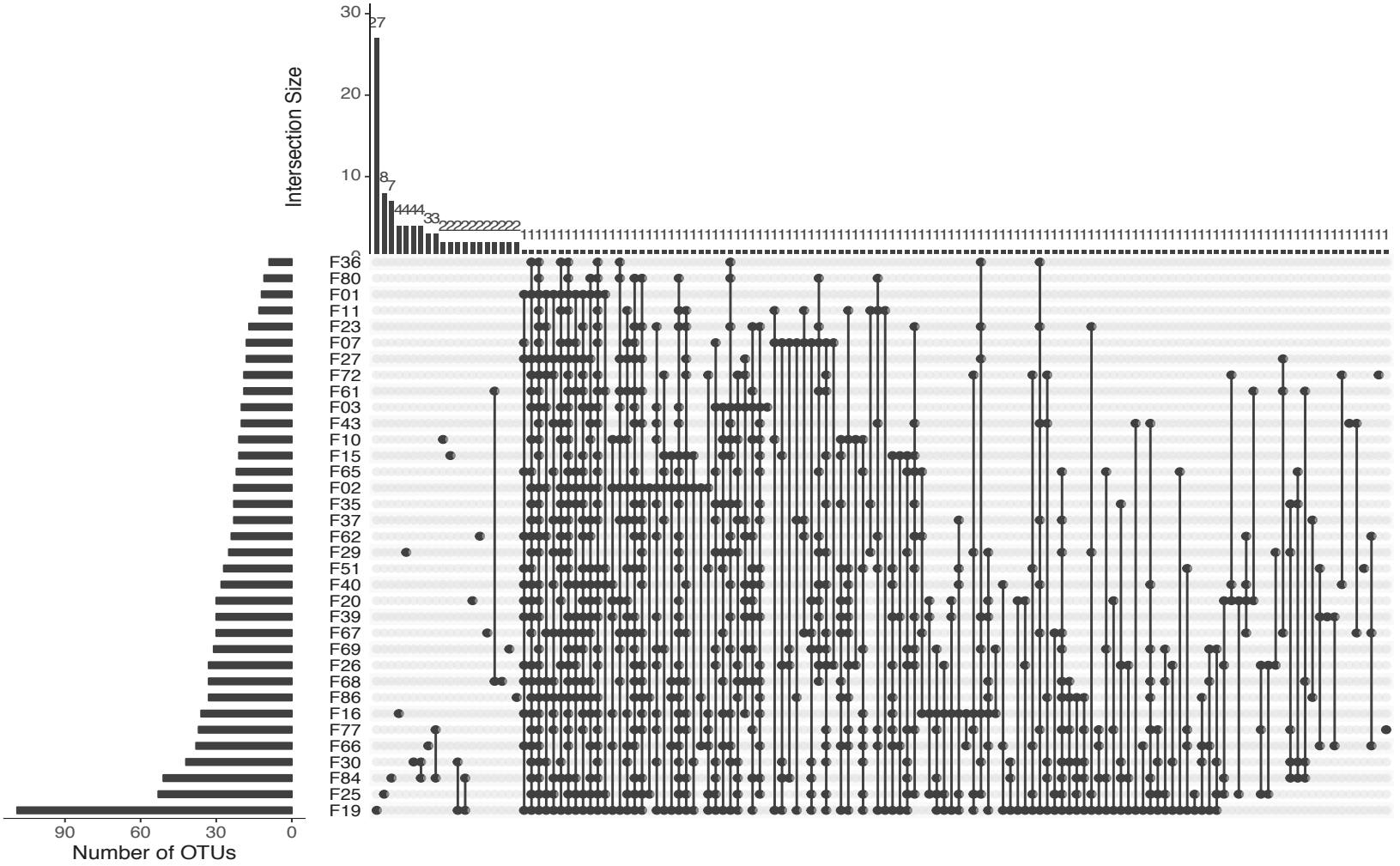
OTU	Dissimilarity (%)	RG2 mean	RG3 mean
Pseudomonas 2034	1.861	0.535	2.05
Uncultured bacterium *Mycoplasma* 3150	1.547	1.63	0.891
Uncultured bacterium *Psychrobacter* 1488	1.501	0.634	1.35
Ambiguous taxa *Maribacter* 1117	1.263	1.37	1.29
Uncultured bacterium *Rhodobacteraceae* 6466	1.196	0.965	1.13
Rhodospirillaceae 6418	1.083	0.721	0.926
Uncultured bacterium *Mycoplasmataceae* 680	1.07	1.09	0.68
Ambiguous taxa *Francisella* 2095	0.9425	0.991	1.14
Winogradskyella 1511	0.8938	1.33	1.1
Uncultured bacterium *Rhodobacteraceae* 6466	0.841	0.872	0.903



Supplementary Figure 3.2 The total *Vibrio* load determined with a *Vibrio* specific 16S qPCR assay. Blue columns represent the average *Vibrio* load in a family, while red columns represent the average *Vibrio* load for the resistance group (RG). Error bars are standard error. Statistical comparisons between resistance groups are displayed on the resistance group mean values.

Supplementary Table 3.5 Maximal information-based nonparametric exploration (MINE) analysis of expected breeding values (EBVs).

X variable	Y variable	MIC	Linear regression	p-value
Shell length	Depth index	0.81363	-0.9193912	<0.001
Oyster weight	Shell length	0.59185	0.74213886	0.002
Width index	Meat condition	0.54716	0.44662884	0.006
Width index	Depth index	0.54643	0.7001985	0.006
Width index	Shell length	0.54168	-0.8329716	0.007
Oyster weight	Meat condition	0.49895	-0.3160977	0.02
Disease resistance	Oyster weight	0.45763	0.1452698	0.04
Shell length	Meat condition	0.45451	-0.2803066	0.04
Oyster weight	Width index	0.45087	-0.5962815	0.04
Disease resistance	Width index	0.44843	-0.3397912	0.05



Supplementary Figure 3.3 Upset figure showing core microbes in each oyster family. Numbers of OTUs are displayed in a column chart. Unconnected dots represent unique OTUs only seen in that family line. Connected dots represent OTUs that are present in more than one family line. UpSet figure was made using the UpSetR package (Conway et al., 2017) in the R statistical environment (R_Core_Team, 2017).

Chapter Four

Multiscale heterogeneity in the Pacific oyster microbiome

Chapter Four - 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 manuscript has been formally submitted and is formatted to adhere to the specific formatting requirements of FEMS Microbiology Ecology.

King, W.L., Siboni, N., Kahlke, T., Dove, M., O'Connor, W., Mahbub, K.R., Jenkins, C., Seymour, J.R., Labbate, M. Multiscale heterogeneity in the Pacific oyster microbiome. Submitted.

Publication status: Under review

Supplementary information is provided following the acknowledgments section

Date: 16/09/19

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

4.1 Summary

Different tissues and organs of a host macroorganism can represent distinct microenvironments resulting in the establishment of multiple discrete microbiomes within an individual host. For the Pacific oyster, *Crassostrea gigas*, the factors governing the bacterial composition of different oyster microenvironments (oyster tissues) are poorly understood. In this study, the bacterial microbiomes associated with the *C. gigas* mantle, gill, adductor muscle, and digestive gland were characterised using 16S (V3-V4) rRNA amplicon sequencing across six discrete estuaries. Both location and host tissue-type, with tissue-type being the stronger determinant, were factors in driving microbiome composition. Microbiomes from wave-dominated estuaries displayed similar compositions and had significantly elevated 16S rRNA gene counts despite being geographically distant from one another, possibly indicating that functional estuarine morphology characteristics are a factor shaping the oyster microbiome. Despite the multiscale oyster microbiome heterogeneity, examinations of the core microbiome identified uncultured *Spirochaetaceae* taxa as being highly conserved across all sites and sampling tissues, while members of the *Vulcaniibacterium*, *Spirochaetaceae* and *Margulisbacteria*, and *Polynucleobacter* were regionally conserved members of the digestive gland, gill, and mantle microbiomes respectively. These results indicate that a baseline microbiome profile for specific locations is necessary when investigating a role for microbiomes in oyster health.

4.2 Introduction

In many marine plants and animals, the microbiome is an important determinant of host health and physiology (Rosenberg et al., 2007; Tarnecki et al., 2017; Crump et al., 2018; Pita et al., 2018). However, it is increasingly apparent that the structure and influence of the microbiome is dynamic with multiple factors including diet (Wilkes Walburn et al., 2019), location (Cúcio et al., 2016) and time (Kimes et al., 2013) driving heterogeneity.

Shifts in microbiome composition occurs in a number of marine benthic organisms including tubeworms (Vijayan et al., 2019), corals (Woo et al., 2017; Marcelino et al., 2018) and seagrasses (Cúcio et al., 2016) and, is driven by broad-scale external processes such as seasonal changes (Sharp et al., 2017), geographic location (Cúcio et al., 2016; Woo et al., 2017) and time (Vijayan et al., 2019). Despite the apparent inherent variability within microbiomes, taxa are often conserved over large geographic scales (Ainsworth et al., 2015) and time periods (Aronson et al., 2017).

Within an individual, a host organism's microbiome can vary (Ainsworth et al., 2015; Marcelino et al., 2018) due to distinct microenvironments associated with different host tissues or organs (Ainsworth et al., 2015; Crump et al., 2018; Marcelino et al., 2018). These host-associated (individual-scale) microenvironments may accommodate a range of ecological interactions between the host organism and its microbial consortia (Jensen et al., 2007; Ainsworth et al., 2015; Brodersen et al., 2018; Marcelino et al., 2018), important for key physiological processes such as nitrogen fixation in seagrasses (Lehnen et al., 2016), nutrient uptake in corals (Ainsworth et al., 2015) and seagrasses (Harlin, 1973), and host defences in tunicates and sponges (reviewed by (Florez et al., 2015)). Therefore, broad-

scale and individual-scale processes act in concert to shape the host microbiome.

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

Understanding the factors governing the structure of the *C. gigas* microbiome is important because there is evidence suggesting that the microbiome is a factor in oyster disease dynamics (Wegner et al., 2013; Lokmer and Wegner, 2015; De Lorgeril et al., 2018; Green et al., 2019; King et al., 2019a). While these studies have provided evidence that the oyster microbiome may be intimately involved in polymicrobial infection dynamics (Petton et al., 2015; De Lorgeril et al., 2018), there are limited culture-independent studies examining the microbiome of *C. gigas* without the

confounding influence of disease (Lokmer et al., 2016a; Lokmer et al., 2016b). Identifying a 'healthy' or 'normal' baseline microbiome and defining the dynamics (e.g. physiological and environmental factors) that influence its structure is essential when aiming to interpret its role in health including susceptibility to pathogens.

To define the baseline *C. gigas* microbiome and in doing so consider the extent of microbiome heterogeneity between different oyster tissues (oyster microenvironments) and different regions, we examined the microbiome of four different oyster tissues across six different estuaries spanning four degrees of latitude along the eastern coastline of Australia (New South Wales, Australia). Our principal goals were to understand the extent of heterogeneity versus conservation of the *C. gigas* microbiome across different spatial scales, including between different oyster microenvironments (tissue types) and across biogeographically disparate environments. This study provides a necessary foundation for disentangling the role of the microbiome in oyster health and in resolving its role in oyster disease.

4.3 Methods

4.3.1 Oyster collection sites and sampling

To examine the spatial heterogeneity of oyster microbiomes, *C. gigas* samples were collected from six oyster farms along the east coast of New South Wales (NSW), Australia (Figure 4.1), spanning a distance of approximately 470 kilometres. Starting from the southernmost location, the sampled environments included: The Wapengo lagoon, Clyde River, Shoalhaven River (Crookhaven river), Georges River, Hawkesbury River, and Port Stephens. The Clyde River is the largest producer of *C. gigas*

representing 41 % of all oysters produced in NSW, followed by Port Stephens (27 %), the Hawkesbury River (9 %), and the Shoalhaven River (9 %) (DPI, 2019). All sampling locations are tide-dominated drowned valley estuaries (Roy et al., 2001), except for the Wapengo and Shoalhaven sites, which are wave-dominated barrier estuaries (Roy et al., 2001).

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

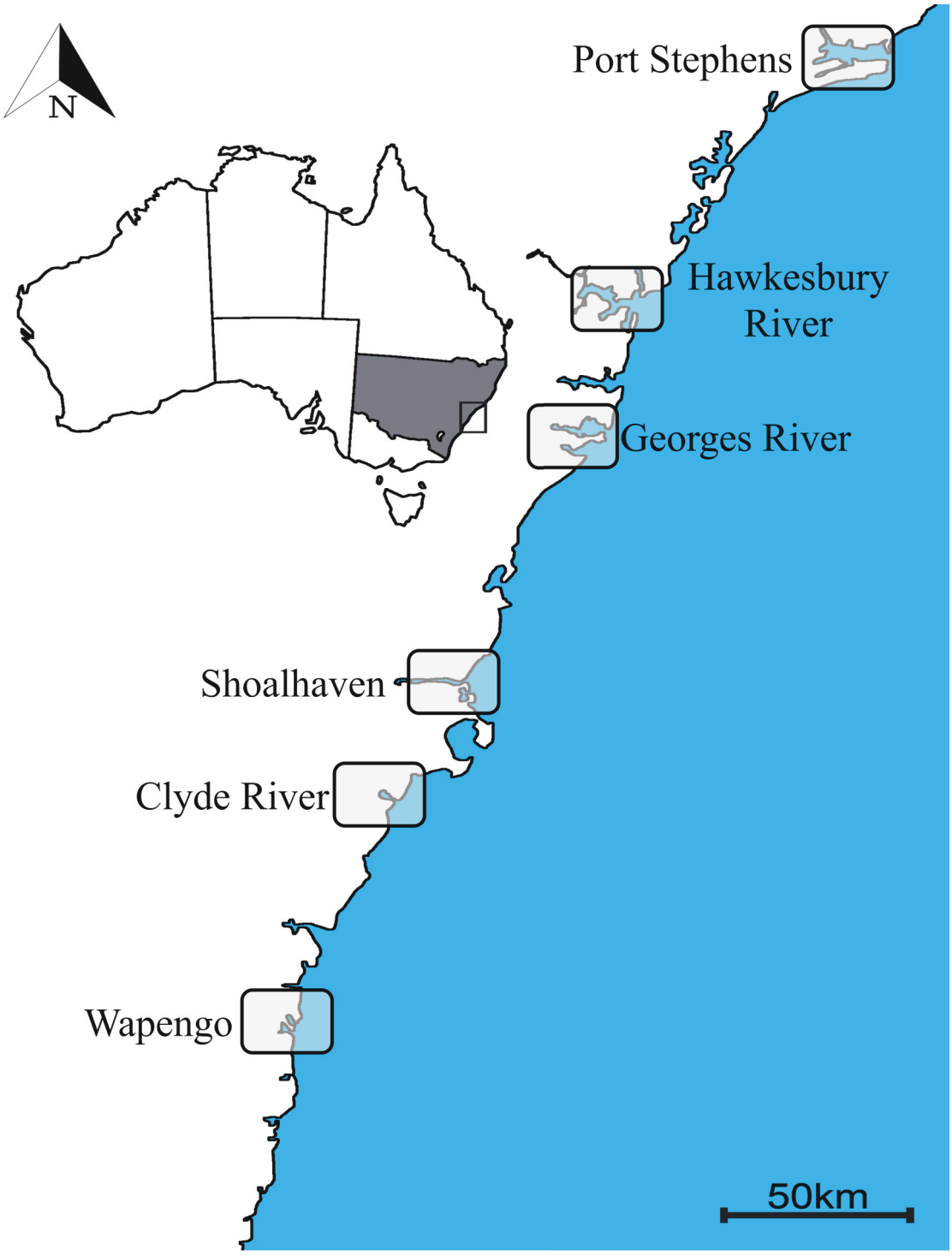


Figure 4.1: Sampling locations across New South Wales, Australia.

4.3.2 Extraction of DNA from different oyster tissues

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

4.3.3 Quantitative PCR (qPCR)

To provide an indication of the bacterial abundance within each sample, we employed a quantitative PCR (qPCR) assay to quantify total 16S rRNA gene copies. An epMotion 5075l Automated Liquid Handling System integrated with a Bio-Rad CFX384 Touch Real-Time PCR Detection System was used to perform the analysis. All analyses were performed with three technical replicates with a standard curve and negative controls, using the following reaction mixture: 2.5 μL iTaq Universal SYBR Green supermix, 0.2 μL of each 10 μM forward and 10 μM reverse primer, 1 μL of template DNA, and 1.1 μL of sterile water. Bacterial abundance was quantified using the 16S rRNA specific primers BACT1369F (CGGTGAATACGTTCYCGG) and PROK1492R (GGWTACCTTGTTACGACTT) (Suzuki et al., 2000). The qPCR cycling conditions were: 95 °C for 3 minutes followed by 45 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds. The resulting data were normalised to tissue weight. A coefficient of variation (CV) was then calculated for the technical triplicates, and where necessary, samples with CV > 2 % had a replicate removed from the analysis. A melting

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

4.3.4 Oyster microbiome analysis

The microbial community composition of each oyster tissue was characterised with 16S rRNA amplicon sequencing, using the 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primer pair (Herlemann et al., 2011) targeting the V3-V4 region of the 16S rRNA gene. The PCR cycling conditions generating the 16S rRNA amplicons were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Sequencing was performed using the Illumina MiSeq platform at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia). Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA551083.

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME 2 version 2018.6.0) pipeline (Bolyen et al., 2018). Briefly, paired-ended 16S DNA sequences were imported using the 'qiime tools import' command. Sequences were then trimmed and denoised using DADA2 version 1.6, which also removes chimeras (Callahan et al., 2016). Taxonomy was then assigned at the single nucleotide level using the classify-consensus-vsearch qiime feature classifier against the Silva v132 database (Quast et al., 2013). Sequences identified at the single nucleotide threshold are henceforth denoted as ZOTUs (zero-radius OTUs). For those ZOTUs with poor taxonomic assignment, the representative sequence was blasted against the NCBI database. The dataset was further cleaned by removing ZOTUs with less than 400 reads and those identified as

chloroplasts or mitochondria. Cleaned data were then rarefied at 8,100 reads per sample corresponding to a threshold that permitted the inclusion of 5 or more replicate samples for every tissue type (217 remaining samples).

4.3.5 Core microbiome analysis

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

4.3.6 Statistical analysis

Alpha diversity measures, including species diversity (Shannon's index), species evenness, and species richness (observed species) were calculated in the QIIME 2 statistical environment and compared using a Kruskal-Wallis test. To compare community structure between sampling locations and tissue types, normalised data (square root (x)) were first compared using non-metric multidimensional scaling analysis (nMDS). Microbial assemblages were subsequently compared using a one-way PERMANOVA to elucidate significant microbiome patterns across tissue types and sampling locations. To identify which bacterial taxa were driving the difference between locations and tissue types, a similarity percentage analysis (SIMPER) with a Bray-Curtis similarity index was used with data summarised at the genus level. Comparisons of 16S rRNA gene copies (16S rRNA qPCR) were first performed with a Kruskal-Wallis statistical test followed by a Mann-Whitney pairwise test. All beta diversity (nMDS, PERMANOVA, and

SIMPER) and qPCR comparisons were performed in the PAST statistical environment (Hammer et al., 2001).

4.4 Results

4.4.1 Patterns in Bacterial abundance inferred from 16S rRNA qPCR

Estimations of bacterial abundance, as determined by qPCR of the 16S rRNA gene, were highest in oysters collected from the Shoalhaven and Wapengo sampling locations (Figure 4.2; Supplementary Table 4.1) and differed significantly between the other sampling locations ($H = 87$; $p < 0.0001$). Overall, the mantle tissue had the highest number of 16S rRNA gene copies compared to all other tissues ($H = 40$; $p < 0.0001$; Supplementary Table 4.2) with the mantle and gill tissues at each sampling location generally having higher 16S rRNA gene copies compared to the adductor muscle and digestive gland (Supplementary Table 4.3). The mantle and gill samples from Wapengo and Shoalhaven both had consistently higher numbers of 16S rRNA gene copies compared to all other tissues at all other sites except for the adductor muscle tissue at Shoalhaven (Supplementary File 2).

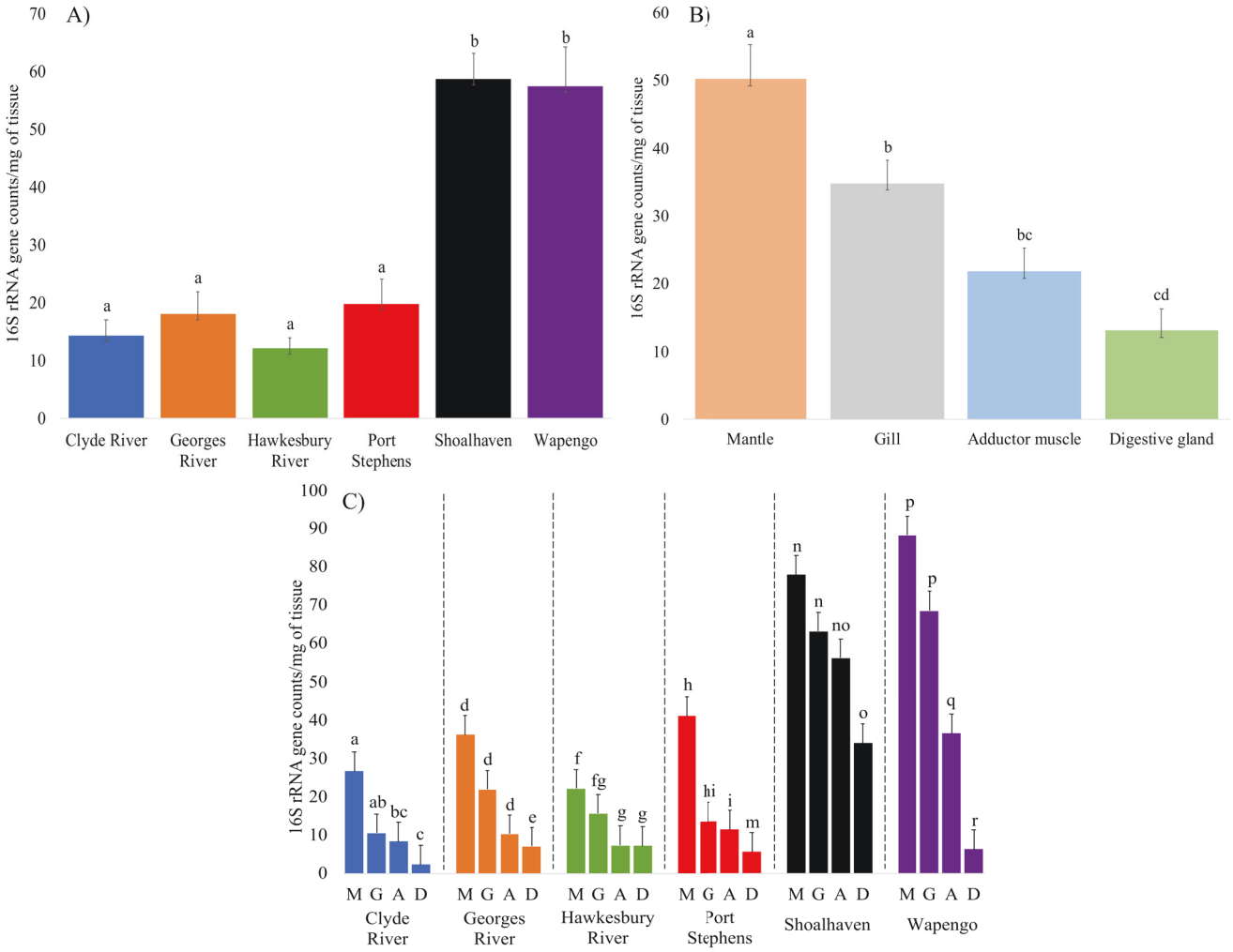


Figure 4.2: 16S rRNA gene copy number for oysters and their tissues. Data are average 16S rRNA counts per milligram of tissue with standard deviation. A) 16S rRNA counts per location. B) 16S rRNA counts per tissue type. C) 16S rRNA counts for each tissue at each location. Significant comparisons are denoted by different letters. Comparisons for section C) were performed within locations.

4.4.2 Alpha diversity comparisons

Species richness, evenness, and diversity were all significantly different between sampling locations (richness H = 98, p = < 0.0001; evenness H = 32, p = < 0.0001; diversity H = 70, p = < 0.0001). Oyster microbiomes from the Wapengo and Shoalhaven locations displayed the greatest levels of species richness and diversity (Supplementary Table 4.4; Supplementary File 2). Oyster microbiomes from the Wapengo, Shoalhaven, and Hawkesbury River locations had the greatest species evenness.

All measured alpha diversity indices were also significantly different between tissue-types (richness H = 24, p = < 0.0001; evenness H = 17, p = 0.0008; diversity H = 28, p = < 0.0001). The digestive gland microbiome displayed the greatest levels of species richness (Supplementary Table 4.5). Species evenness was consistent across the gill, adductor muscle, and digestive gland, but was significantly lower in the mantle. Similarly, the mantle tissue had the lowest levels of species diversity, with highest diversity levels within the digestive gland and gill (Supplementary Table 4.5).

4.4.3 Geographic location and tissue type are significant determinants of the C. gigas microbiome

The structure of the *C. gigas* microbiome differed significantly according to both sampling location (F = 11; p = 0.0001) and the tissue type (F = 13.6; p = 0.0001) (Table 4.1 and 4.2). However, despite these statistical differences, clear partitioning of the microbial assemblages was not evident when all data was included in an nMDS analysis (stress 0.28).

Table 4.1: One-way PERMANOVA pairwise comparisons between sampling locations, including the p-value and F-value

Location	Clyde river	Georges river	Hawkesbury river	Port Stephens	Shoalhaven
Georges river	0.0002 (3.9)				
Hawkesbury river	0.0001 (6.2)	0.0001 (4.7)			
Port Stephens	0.0001 (5.3)	0.028 (2)	0.0009 (3.6)		
Shoalhaven	0.0001 (23.9)	0.0001 (19.9)	0.0001 (13.1)	0.0001 (18.3)	
Wapengo	0.0001 (18.9)	0.0001 (15.7)	0.0001 (10.9)	0.0001 (14.8)	0.0058 (2.6)

Table 4.2: One-way PERMANOVA pairwise comparisons between tissue types, including the p-value and F-value

Tissue type	Mantle	Gill	Adductor muscle
Gill	0.0001 (12.7)		
Adductor muscle	0.0001 (8.7)	0.0001 (9.5)	
Digestive gland	0.0001 (27)	0.0001 (15.8)	0.0001 (8.4)

To further resolve the influence of tissue type or sampling environment on the oyster microbiome structure, we compared the microbiomes of different tissue types within, and between, sampling environments. In the first instance, tissue-specific oyster microbiomes differed significantly to each other within all locations (Figure 4.3; Clyde River $F = 4.5$, $p = 0.0001$; Georges River $F = 5$, $p = 0.0001$; Hawkesbury River $F = 3.6$, $p = 0.0001$; Port Stephens $F = 5.2$, $p = 0.0001$; Shoalhaven $F = 3.9$, $p = 0.0001$; Wapengo $F = 3.9$, $p = 0.0001$). Notably, significant differences occurred between all pairwise comparisons between tissues at all sites (Supplementary File 2), implying a strong tissue-type influence on the oyster microbiome.

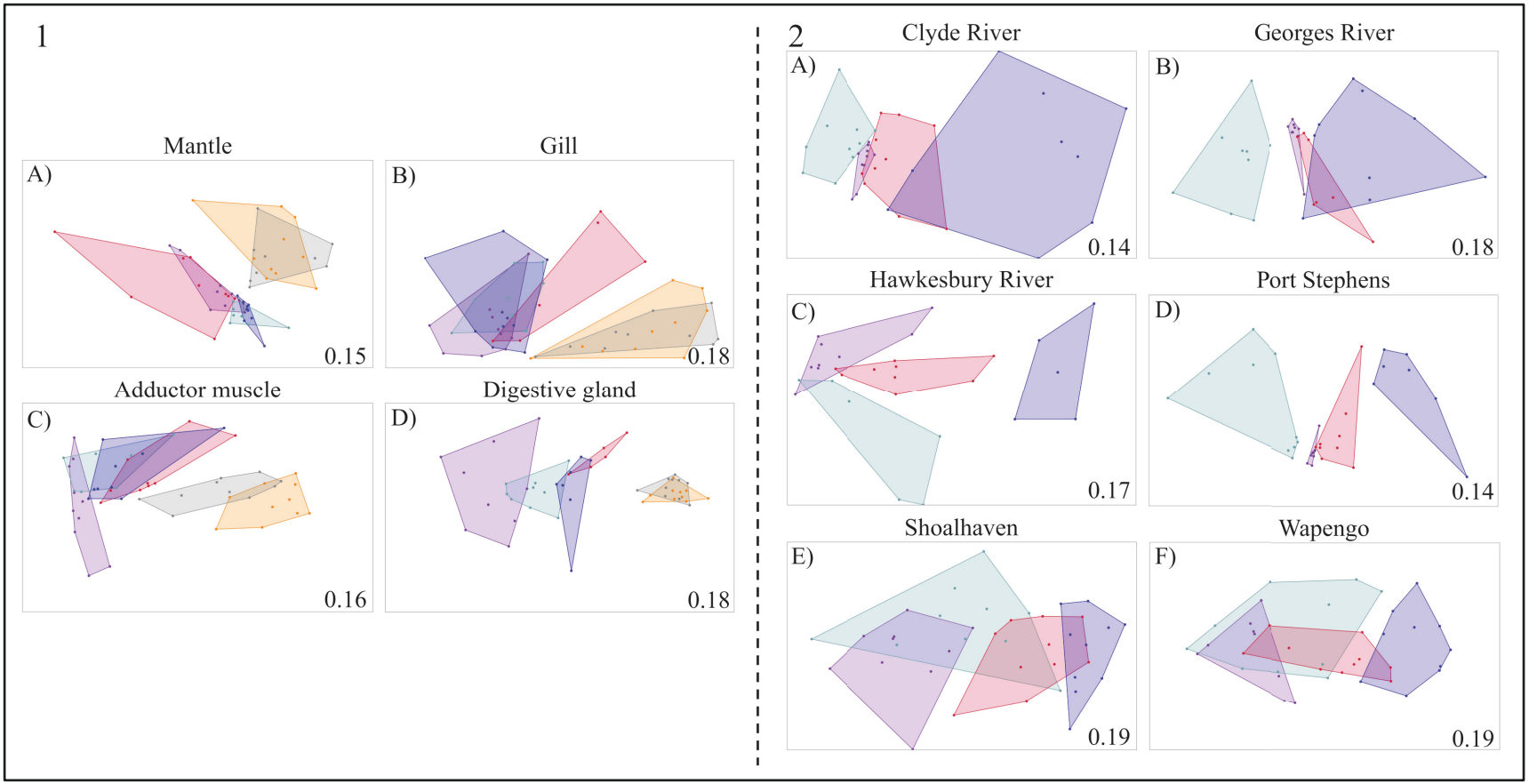


Figure 4.3: nMDS plots of oyster microbiomes for individual tissues across locations (panel 1; left) and for different tissues within locations (panel 2; right). For panel 1: Clyde River = purple, Hawkesbury River = red, Georges River = green, Port Stephens = blue, Shoalhaven = orange, and Wapengo = grey. For panel 2: Mantle tissues = purple, gill tissues = green, adductor muscle tissues = red, and digestive gland tissues = blue. Stress values are provided in the lower right corner of each subfigure.

When using data summarised at the genus level, uncultured *Spirochaetaceae* bacteria were the strongest driver of tissue-specific microbiome differences within sites (Figure 4.4), contributing 10.7 % to the dissimilarity between tissues (Table 4.3), primarily due to their over-representation in the mantle tissue. Members of the *Mycoplasma* and *Vulcaniibacterium* genera were responsible for 6.1 % and 4.8 % of the dissimilarity contribution between tissues, primarily due to an overabundance of these genera in the digestive gland. Members of the *Spirochaetaceae* family and the *Margulisbacteria* phylum were responsible for 2.4 %, and 2.1 % of the microbiome variability between tissues respectively, predominantly due to their over-representation in the gill tissue. Members of the *Acidovorax* genus accounted for 4.5 % of the microbiome dissimilarity between tissues and were most abundant in adductor muscle and digestive gland microbiomes. The *Polynucleobacter* genus accounted for 3.4 % of the dissimilarity contribution between tissue-types and were most abundant in the mantle.

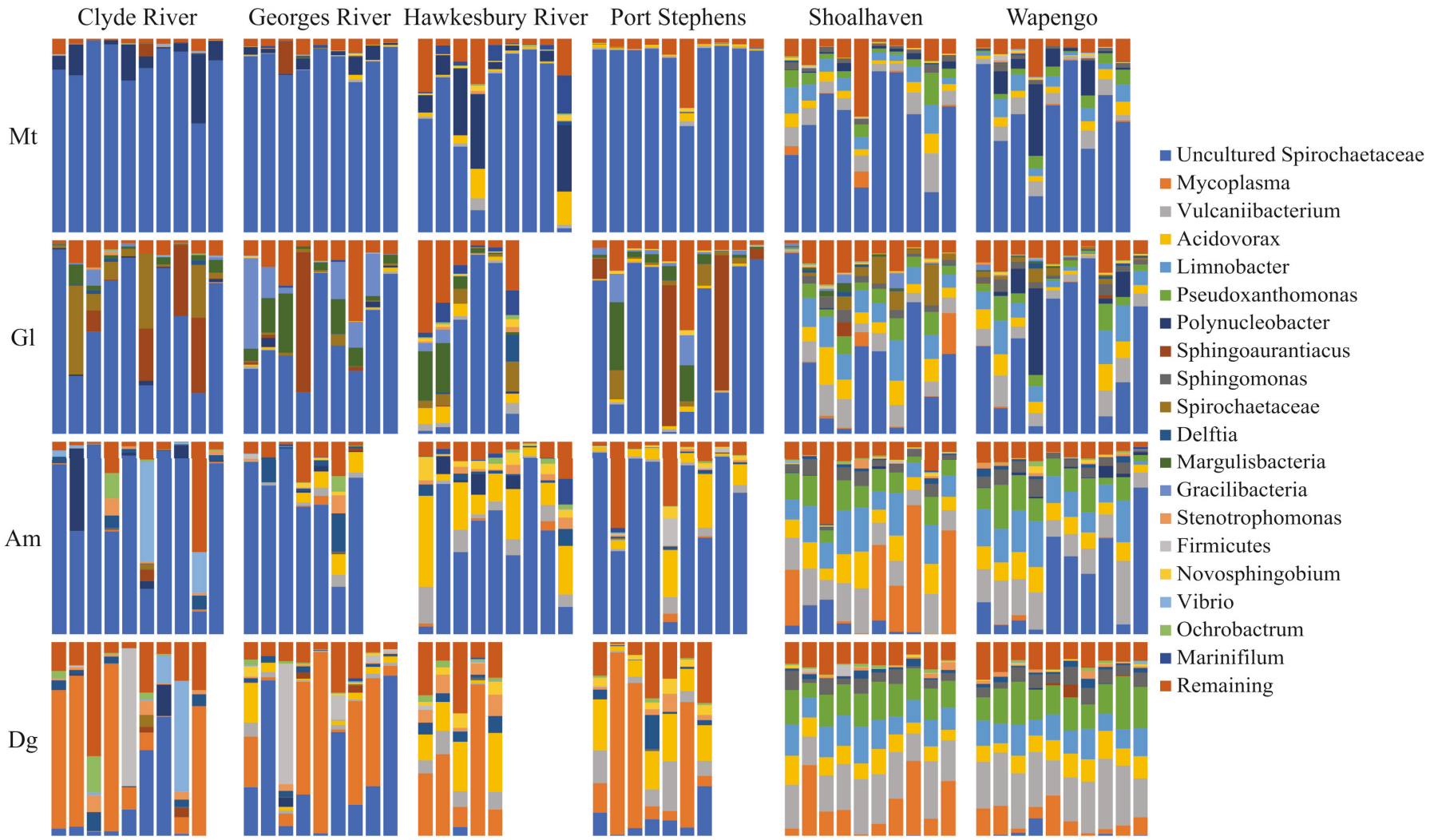


Figure 4.4: Summarised oyster microbiomes at the genus level, across six sampling locations and four sampled tissues. Tissues are labelled as: Mt = mantle, Gl = gill, Am = adductor muscle and Dg = digestive gland. Top 20 summarised genera are shown with the remaining genera grouped together (other).

Table 4.3: SIMPER analysis between grouped tissue types using data summarised to the genus level, including the taxa, dissimilarity contribution, and the mean abundance for each tissue-type (%).

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

Tissue-specific microbiomes differed significantly between sampling locations (Figure 4.3; Mantle F = 5.3, p = 0.0001; Gill F = 4, p = 0.0001; Adductor muscle F = 5.3, p = 0.0001; Digestive gland F = 4.4, p = 0.0001), further confirming the regional-scale spatial variability of the *C. gigas* microbiome composition. However, when examining pairwise comparisons of specific tissues between individual locations, the mantle, gill, and digestive gland microbiomes from Wapengo were not significantly different to the same tissue types at the Shoalhaven site (Supplementary File 2). These similarities are perhaps notable, given that the Wapengo and Shoalhaven sites are the only two sampled sites characterised as wave-dominated estuaries. Pairwise comparisons of adductor muscle microbiomes from the tide-dominated estuaries were not significantly different between the Georges River site when compared to the Clyde River and Port Stephens sites. Further, the adductor muscle microbiome from the Hawkesbury River were not significantly different to those from Port Stephens. These data suggest a regional influence on the oyster microbiome composition, though it is notable that these large-scale differences in microbiome structure were not as strong as the microenvironmental-scale, tissue-type influence.

Uncultured *Spirochaetaceae* bacteria contributed to the greatest dissimilarity between oyster microbiomes from different sampling locations, accounting for 10 % of the variability between sites, largely due to a relative over-abundance of these bacteria in the Clyde River, Georges River, Hawkesbury River, and Port Stephens (Table 4.4). Bacteria assigned to the *Vulcaniibacterium* genus were responsible for 5.2 % of the microbiome variability between sites, driven by an over-representation in the Wapengo and Shoalhaven sampling location microbiomes. At these sites, members of the *Limnobacter* and *Pseudoxanthomonas* genera were also over-represented, contributing 4.5 % and 4.1 % to the microbiome dissimilarities,

while they were completely absent, or in low abundance, at the other four sampling locations. Bacteria assigned to the *Vibrio* genus were over-represented in the adductor muscle and digestive gland microbiomes at the Clyde River site, relative to all other locations, contributing 1.1 % of the dissimilarity between microbiomes. Members of the SAR11 clade contributed 1 % to the dissimilarity between sites and were common across the Clyde River, Georges River, Hawkesbury River, and Port Stephens sites but were almost completely absent in the Wapengo and Shoalhaven sites.

Table 4.4: SIMPER analysis between grouped sampling locations using data summarised to the genus level, including the taxa, dissimilarity contribution, and the mean abundance (%) for that location. CR is Clyde river, GR is the Georges river, HR is the Hawkesbury river, SH is the Shoalhaven site, PS is the Port Stephens site, and WA is the Wapengo site.

Taxa	Dissimilarity contribution (%)	CR mean	GR mean	HR mean	PS mean	SH mean	WA mean
Uncultured *Spirochaetaceae* family	9.966	59.1	60.4	43.4	59.6	24.5	33
Mycoplasma genus	5.371	8.64	9.03	6.36	7.54	10.7	2.34
Vulcaniibacterium genus	5.164	0	1.26	4.38	2.89	13.7	14.3
Acidovorax genus	4.71	0	2.51	11.3	6.06	8.28	7.39
Limnobacter genus	4.481	0	0	0	0.014	12.1	10.6
Pseudoxanthomonas genus	4.133	0	0	0	0.001	9.78	10
Polynucleobacter genus	3.347	4.6	1.48	5.74	0	0.47	4.5
Sphingomonas genus	2.714	0.053	0.032	0.056	0.045	4.03	4.15
Delftia genus	2.268	1.63	1.67	2.39	1.07	0.95	1.14
Vibrio genus	1.231	4.03	0.074	0.10	0.073	0.075	0.14
SAR11 clade	1.014	0.19	0.208	0.47	0.56	0.015	0.006

4.4.4 Conservation of the C. gigas core microbiome

As the structure of the oyster microbiome was governed by both the sampling location and tissue type, we sought to identify core microbiomes for (i) all of the tested oyster microbiomes (universal core microbiome), (ii) each sampling location, and (iii) each tissue type (Figure 4.5). When including all samples in the core analysis, several ZOTUs assigned to an uncultured *Spirochaetaceae* (ZOTUs 04655, 29fe1, fe651, 3bb6f, 295f6, 80d03, 1986e, and a9435) were characterised as members of the 'universal' core microbiome, whereby they were found in at least 80% of all tested samples, regardless of sampling location or tissue type.

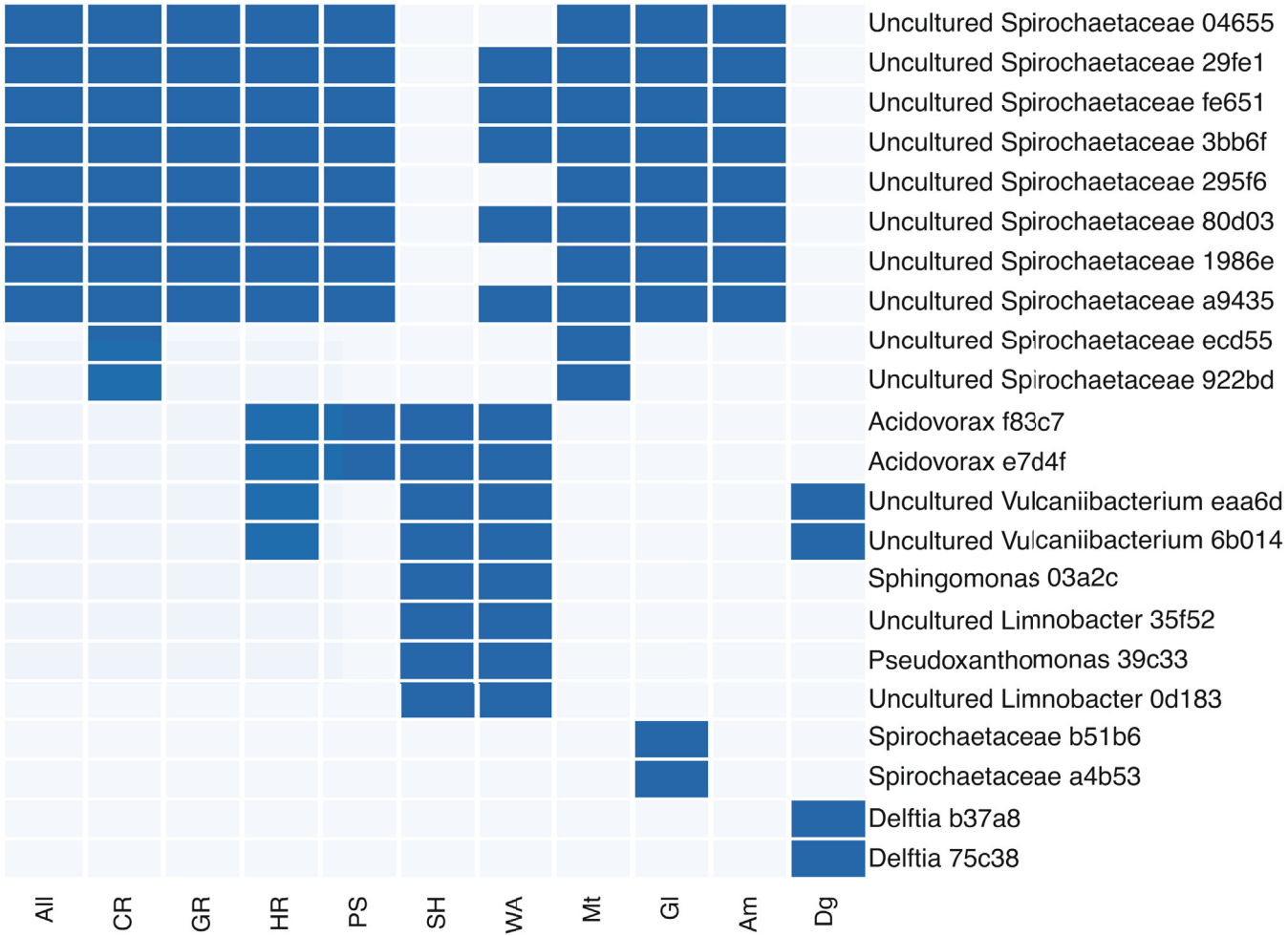


Figure 4.5: Presence/absence heatmap of taxa identified as the core microbiome. Dark blue boxes represent the presence of a core taxa. Core microbiome analyses were performed using i) all samples regardless of location or tissue, ii) individual sites regardless of tissue and iii) individual tissues regardless of location. All = All samples were included in the analysis, CR = Clyde River, GR = Georges River, HR = Hawkesbury River, PS = Port Stephens, SH = Shoalhaven, and WA = Wapengo. Mt = mantle, Gl = gill, Am = adductor muscle, Dg = digestive gland.

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

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

4.5 Discussion

4.5.1 Both location and tissue-type influence the oyster microbiome assemblage

Geographic location has previously been found to influence the haemolymph, mantle, gill (Lokmer et al., 2016a; Lokmer et al., 2016b), and disease-affected adductor muscle microbiomes (King et al., 2019a). Consistent with these studies, we observed a significant effect of location on the oyster microbiome. However, microbiome similarities between the

mantle, gill, and digestive gland microbiomes from Shoalhaven and Wapengo locations (wave-dominated estuaries), and between the adductor muscle microbiomes at the remainder of the sampling locations (tide-dominated estuaries) over large geographic distances, suggests that geographic location is only one factor driving heterogeneity in the microbiome. These data suggest that estuary-type influences the microbiome composition and should be considered when examining patterns in microbiome heterogeneity between individuals. The oyster microbiome assemblage was also influenced by the oyster tissue, with each tissue harbouring a unique microbial consortia, as previously observed (Lokmer et al., 2016b). This pattern was observed for all pairwise comparisons within all locations, suggesting that tissue-type is a stronger driver of microbiome composition than geographic location.

4.5.2 Estuary properties and their potential influence on the oyster microbiome

Similarities between the microbiomes from the Wapengo and Shoalhaven sites were surprising, given the distance between sampling sites (approximately 200 km). These two sites shared a core microbiome not observed in any other sampling locations, and displayed no significant microbiome differences between the mantle, gill, and digestive gland microbiomes. With members of the *Vulcaniibacterium*, *Limnobacter* and *Pseudoxanthomonas* genera representing the predominate taxa driving the differences between both Wapengo and Shoalhaven, and the other four sampling locations. The Shoalhaven site has a catchment size of 7, 500 km^2 (Roy et al., 2001), with approximately 35 % of the catchment used for agricultural purposes (OceanWatch-Australia, 2017). In contrast, Wapengo has a significantly smaller catchment of 73 km^2 (Roy et al., 2001) but a similar level of agricultural usage at 20 % (OceanWatch-Australia, 2010).

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

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

site were markedly over-represented by *Vibrio* bacteria when compared to all other locations. Given the reduced growth rate and lower nutrient loads in the Clyde River, these could act as a stressor on the oyster allowing *Vibrio* bacteria to colonise and proliferate (Lemire et al., 2015; Bruto et al., 2017; De Lorgeril et al., 2018; King et al., 2019c).

4.5.3 Oyster tissue microbiome heterogeneity

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

dominance with bacteria assigned to the *Spirochaetaceae* family in the oyster gill microbiome. Bacteria assigned to the *Polynucleobacter* genus and an uncultured *Spirochaetaceae* were over-represented in the mantle. *Polynucleobacter* species have previously been observed in oyster homogenate microbiomes (Fernandez-Piquer et al., 2012), this genus contains both obligate endosymbionts of ciliates (Heckmann and Schmidt, 1987; Vannini et al., 2005) and planktonic bacteria (Hahn et al., 2010). Finally, members of the *Acidovorax* genus were over-represented in the adductor muscle and digestive gland microbiomes. Members of the *Acidovorax* have been isolated from a diverse range of environments including soil (Chaudhary and Kim, 2018), water (Pal et al., 2018), and from cyanobacterial blooms (Chun et al., 2017) and could be a food-associated microbe.

4.5.4 Conservation of Spirochaete ZOTUs across sampling environments and tissue types

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

of the *Brachyspiraceae* family. This discrepancy is likely attributed to previously using the Greengenes database for taxonomy assignment, as opposed to the SILVA database in this study. We also previously found it associated with OsHV-1 disease-resistant oysters (OTU 4737 in (King et al., 2019c)). Apart from its presence in different oyster microbiome datasets across different countries and locations within Australia, little is known about these bacteria. Future studies should attempt to further phylogenetically characterise these bacteria and identify their potential functional role(s) within *C. gigas*.

4.6 Conclusions

Emerging evidence suggests that the oyster microbiome is dynamic, shaped by a range of broad- and individual-scale processes however, elements such as estuarine morphology characteristics have yet to be considered as influencing the microbiome. Our analysis revealed that the structure of the *C. gigas* microbial assemblage is governed by both geographic location and tissue type, with microbiomes derived from wave-dominated estuaries exhibiting similar microbiome assemblages despite large geographic separation, with a predominance of soil/particulate-associated bacteria within these microbiomes. Given the dynamic nature of oyster microbiomes, our understanding of whether the oyster microbiome has conserved elements across regions or microenvironments is lacking. We revealed a core microbiome within individual tissue-types, and a universal core microbiome consisting of uncultured *Spirochaetaceae*, as conserved across all sampling locations and tissue types. This finding was strengthened by the presence of this taxa in other previously published oyster microbiome datasets. Due to the dynamic nature of the microbiome, and the strong effect of location and tissue-type on the oyster microbiome, it is difficult to interpret disease-

affected microbiomes based on oyster microbiomes from different locations or tissues. Instead, future studies should aim to characterise the healthy microbiomes of oysters for the specific location where oysters are grown to use as a reference during disease events.

4.7 Acknowledgments

This research was supported by an Australian Research Council Linkage Project (LP160101785) to JRS and ML; 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.

4.8 Supplementary Information

Supplementary Table 4.1: Mann-Whitney pairwise comparisons between sampling locations for bacterial loads as determined by a 16S rRNA-specific qPCR assay.

Location	Clyde river	Georges river	Hawkesbury river	Port Stephens	Shoalhaven
Georges river	0.35 (575)				
Hawkesbury river	0.89 (614)	0.40 (676)			
Port Stephens	0.47 (564)	0.84 (739)	0.43 (645)		
Shoalhaven	<0.001 (93)	<0.001 (175)	<0.001 (79)	<0.001 (150)	
Wapengo	<0.001 (175)	<0.001 (190)	<0.001 (190)	<0.001 (244)	0.53 (641)

Supplementary Table 4.2: Mann-Whitney pairwise comparisons between tissue types for bacterial loads as determined by a 16S rRNA-specific qPCR assay.

Tissue type	Mantle	Gill	Adductor muscle
Gill	<0.001 (1273)		
Adductor muscle	<0.001 (776)	0.39 (1983)	
Digestive gland	<0.001 (304)	0.008 (858)	0.057 (663)

Supplementary Table 4.3: Mann-Whitney pairwise comparisons within sampling locations for bacterial loads as determined by a 16S rRNA-specific qPCR assay.

Clyde River	Comparison	p-value (U-value)	Georges River	Comparison	p-value (U-value)
Adductor muscle	Digestive gland	0.056 (6)	Adductor muscle	Digestive gland	0.19 (32)
	Gill	0.079 (23)		Gill	0.31 (36)
	Mantle	0.018 (18)		Mantle	0.002 (8)
Digestive gland	Gill	0.007 (0)	Digestive gland	Gill	0.054 (24)
	Mantle	0.013 (2)		Mantle	<0.001 (4)
Gill	Mantle	0.079 (23)	Gill	Mantle	0.038 (22)
Hawkesbury River	**Comparison**	**p-value (U-value)**	**Port Stephens**	**Comparison**	**p-value (U-value)**
Adductor muscle	Digestive gland	0.97 (49)	Adductor muscle	Digestive gland	0.019 (13)
	Gill	0.10 (28)		Gill	0.62 (43)
	Mantle	0.015 (12)		Mantle	0.045 (23)
Digestive gland	Gill	0.10 (28)	Digestive gland	Gill	0.011 (11)
	Mantle	0.011 (11)		Mantle	0.004 (7)
Gill	Mantle	0.17 (24)	Gill	Mantle	0.10 (28)
Shoalhaven	**Comparison**	**p-value (U-value)**	**Wapengo**	**Comparison**	**p-value (U-value)**
Adductor muscle	Digestive gland	0.12 (29)	Adductor muscle	Digestive gland	0.003 (0)
	Gill	0.62 (43)		Gill	0.031 (21)
	Mantle	0.10 (28)		Mantle	0.002 (8)
Digestive gland	Gill	0.007 (14)	Digestive gland	Gill	0.003 (0)
	Mantle	0.002 (9)		Mantle	0.003 (0)
Gill	Mantle	0.24 (34)	Gill	Mantle	0.12 (29)

Supplementary Table 4.4: Kruskal-Wallis alpha diversity comparisons between sampling locations. Results are presented in the following order; Shannon's diversity index, species evenness, and observed species (species richness). Significant results are also presented with a H-value.

Location	Clyde river	Georges river	Hawkesbury river	Port Stephens	Shoalhaven
Georges river	0.89 0.36 0.23				
Hawkesbury river	0.001 (10.8) 0.03 (4.5) <0.001 (12.3)	0.001 (10.2) 0.003 (8.5) 0.016 (5.8)			
Port Stephens	0.55 0.06 0.041 (4.2)	0.61 0.18 0.40	0.002 (9.6) <0.001 (11.7) 0.062		
Shoalhaven	<0.001 (29.9) 0.022 (5.2) <0.001 (45.5)	<0.001 (26.3) <0.001 (12.9) <0.001 (33.6)	0.07 0.48 <0.001 (23.2)	<0.001 (21.1) <0.001 (10.9) <0.001 (31.9)	
Wapengo	<0.001 (34.6) 0.006 (7.3) <0.001 (43.7)	<0.001 (30.7) <0.001 (16.7) <0.001 (32)	0.036 (4.4) 0.73 <0.001 (20.3)	<0.001 (24.1) <0.001 (13.9) <0.001 (30)	0.93 0.71 0.92

Supplementary Table 4.5: Kruskal-Wallis alpha diversity comparisons between tissue types. Results are presented in the following order; Shannon's diversity index, species evenness, and observed species (species richness). Significant results are also presented with a H-value.

Tissue type	Mantle	Gill	Adductor muscle
Gill	<0.001 (15.1) <0.001 (12.1) 0.004 (8.2)		
Adductor muscle	0.003 (9.1) 0.002 (9.3) 0.18	0.41 0.63 0.31	
Digestive gland	<0.001 (23.5) <0.001 (11) <0.001 (21.8)	0.16 0.72 0.009 (6.9)	0.03 (4.7) 0.52 0.004 (8.2)

Chapter Five

A new high throughput sequencing assay for characterising the diversity of natural *Vibrio* communities and its application on a Pacific oyster mortality event

Chapter Five - 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 manuscript has been formally accepted and is formatted to adhere to the specific formatting requirements of Frontiers in Microbiology.

King, W.L., Siboni, N., Kahlke, T., Green, T.J., Labbate, M., Seymour, J.R. A new high throughput sequencing assay for characterising the diversity of natural *Vibrio* communities and its application on a Pacific oyster mortality event. Frontiers in Microbiology. DOI: 10.3389/fmicb.2019.02907

Publication status: Accepted

Supplementary information is provided following the acknowledgments section

Date: 16/09/19

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

5.1 Abstract

The *Vibrio* genus is notable for including several pathogens of marine animals and humans, yet characterisation of *Vibrio* diversity using routine 16S rRNA sequencing methods is often constrained by poor resolution beyond the genus level. Here, a new high throughput sequencing approach targeting the heat shock protein (*hsp60*) as a phylogenetic marker was developed to more precisely discriminate members of the *Vibrio* genus in environmental samples. The utility of this new assay was tested using mock communities constructed from known dilutions of *Vibrio* isolates. Relative to standard 16S rRNA and *Vibrio*-specific 16S rRNA sequencing assays, the *hsp60* assay delivered high levels of fidelity with the mock community composition at the species level, including discrimination of species within the *V. harveyi* clade, which are often misidentified due to their high genetic similarity. This assay was subsequently applied to characterise *Vibrio* community composition in seawater and delivered substantially improved taxonomic resolution of *Vibrio* species compared to 16S rRNA analysis. Finally, this assay was applied to examine patterns in the *Vibrio* community within oysters during a Pacific oyster mortality event. In these DNA samples, efficacy of the *hsp60* assay was dependent on *Vibrio* abundance, but identified species level *Vibrio* community shifts prior to disease onset in oysters, pinpointing *Vibrio harveyi* as a putative pathogen. Given that shifts in the *Vibrio* community often precede, cause and follow disease onset in numerous marine organisms, including many aquaculture species, there is a need for an accurate high-throughput assay for defining *Vibrio* community composition in natural samples. This *Vibrio*-specific *hsp60* sequencing assay offers the potential for precise high throughput characterisation of *Vibrio* diversity, providing an enhanced platform for dissecting *Vibrio* dynamics in the environment.

5.2 Importance

Vibrio are a marine group of bacteria notable for their inclusion of numerous pathogens of marine animals and humans. Current high throughput sequencing techniques to characterise *Vibrio* diversity provide poor taxonomic classification beyond the genus-level making it difficult to discern *Vibrio* community dynamics and diversity in the environment. In this study, we developed a high throughput sequencing assay for improved species-level characterisation of the *Vibrio* community. In addition, a boutique *Vibrio* reference dataset was created with 106 different *Vibrio* species. This *Vibrio*-specific sequencing assay identified all *Vibrio* species used in a constructed mock community and the assay was successfully applied to examine *Vibrio* diversity in seawater and oysters from a Pacific oyster mortality event. The *Vibrio*-specific sequencing assay represents a substantial new tool to examine *Vibrio* community dynamics in the environment and will be useful for any studies observing fine-scale patterns in the *Vibrio* community.

5.3 Introduction

Vibrio are a genus of gram-negative marine bacteria that are ubiquitous in a number of different aquatic environments, including estuaries, the open ocean, and the deep-sea (Simidu and Tsukamoto, 1985; Thompson et al., 2004; Siboni et al., 2016). The *Vibrio* genus comprises high levels of metabolic diversity and can play important ecological roles in marine biogeochemical cycling (Urdaci et al., 1988; Svitil et al., 1997; Chimetto et al., 2008; Hunt et al., 2008; Grimes et al., 2009) but are most recognised for their often ecologically important relationships with a wide range of aquatic organisms including bivalves, cephalopods, polychaetes, fish, corals, and algae (Lee and Ruby, 1994; Grisez et al., 1997; Hood and Winter, 1997;

Raguenes et al., 1997; Ben-Haim and Rosenberg, 2002; Nyholm and Nishiguchi, 2008; Miyashiro and Ruby, 2012; Lemire et al., 2015; Tout et al., 2015). Notably, while some of these relationships include mutualistic interactions (Huq et al., 1983; Thompson et al., 2006), many are pathogenic with diverse *Vibrio* species causing disease in aquatic animals (Goarant et al., 2000; Kushmaro et al., 2001; Ben-Haim and Rosenberg, 2002; Becker et al., 2004; Frans et al., 2011; Geng et al., 2014; Vezzulli et al., 2015) and, within aquaculture settings resulting in significant economic losses (Lafferty et al., 2015). Notably, some *Vibrio* species are also dangerous human pathogens (Daniels and Shafaie, 2000).

Given their ecological, economic and human health significance, assessing *Vibrio* diversity and the presence of specific *Vibrio* species in the environment is an important objective and a wide suite of both culture-dependent (Donovan and van Netten, 1995; Grisez et al., 1997) and -independent techniques have been applied (Lane et al., 1985; Dorsch et al., 1992). Among culture-independent techniques, 16S rRNA sequencing has been widely used to characterise *Vibrio* community diversity and composition but typically delivers poor species-level resolution, particularly among highly genetically-related *Vibrio* species (Nagpal et al., 1998; Gomez-Gil et al., 2004; Pascual et al., 2010; Cano-Gomez et al., 2011), thereby limiting examination of intra-genus heterogeneity (Poretsky et al., 2014). Previous attempts to employ *Vibrio*-specific 16S rRNA primers have incrementally improved the taxonomic resolution when characterising *Vibrio* diversity (Yong et al., 2006) but the proportion of sequences that can be unambiguously assigned to *Vibrio* species often remains low (Siboni et al., 2016). Due to the inherent inadequacies of the 16S rRNA gene for correctly identifying *Vibrio* species, another gene target encoding the heat shock protein 60 (*hsp60*, also known as *GroEL* or *cpn60*), a type I chaperonin

protein that assists in protein folding (Hemmingsen et al., 1988; Farr et al., 2000; Brinker et al., 2001), has been proposed as a good candidate for *Vibrio* phylogenetic studies (Kwok et al., 2002). Previous studies utilising *hsp60* have primarily relied on *Vibrio* isolates for species identification (Preheim et al., 2011; Szabo et al., 2012; Silvester et al., 2017) but a recent study applied universal *hsp60* primers to characterise *Vibrio* community diversity using high-throughput amplicon sequencing (Jesser and Noble, 2018). While this approach delivered improved *Vibrio* species identification relative to 16S rRNA, it used universal, rather than *Vibrio*-specific, *hsp60* primers and filtered for *Vibrio* assigned species, not unlike 16S rRNA sequencing.

One of the many areas where the development of a high precision assay for determining *Vibrio* community diversity would have great utility is within the aquaculture industry where *Vibrio* infections cause substantial losses in stock and profits (Gay et al., 2004; Lafferty et al., 2015; Lemire et al., 2015; Bruto et al., 2017; Green et al., 2019) but the precise identity of the pathogen is often not well resolved or incorrectly assigned to the wrong species (Sawabe et al., 2013; Richards et al., 2014; Dubert et al., 2017; Green et al., 2019). *Vibrio* species have negative impacts on oyster cultivation by causing mortality in hatchery settings (Sugumar et al., 1998; Takahashi et al., 2000; Elston et al., 2008; Richards et al., 2015; King et al., 2019b). Outside of hatchery settings, a number of *Vibrio* species have been identified as pathogenic towards oysters (Waechter et al., 2002; Wendling et al., 2014; Bruto et al., 2017; Go et al., 2017; King et al., 2019b) with other species implicated in disease events (Saulnier et al., 2010; Duperthuy et al., 2011; Bruto et al., 2017). Because of the complexity of oyster diseases direct causality of *Vibrio* species often remains unproven therefore, they are usually regarded as opportunistic pathogens (Garnier et al., 2007; Jenkins et al., 2013; Go et al., 2017; De Lorgeril et al., 2018). One culture-dependent

study has observed shifts in the *Vibrio* community preceding the onset of disease in oysters, as pathogenic *Vibrio* bacteria replace the natural *Vibrio* commensals (Lemire et al., 2015). To better understand the role of *Vibrio* species in disease events such as those afflicting oysters, a precise high throughput technique that characterises this important bacterial group is required.

Here, a new *Vibrio*-specific *hsp60* amplicon sequencing assay was developed, coupled with a customised *Vibrio hsp60* sequence reference database, designed to precisely characterise *Vibrio* diversity in environmental samples using the Illumina sequencing platform. The utility of this new assay was first validated using mock *Vibrio* communities constructed from varying proportions of *Vibrio* isolates before moving to tests with natural seawater samples, and finally applying it to characterise patterns in *Vibrio* diversity during a Pacific oyster mortality event (Green et al., 2019).

5.4 Methods

5.4.1 Primer and Vibrio reference dataset construction

In order to develop a reference dataset to aid the design of a new set of degenerate primers targeting the *Vibrio hsp60*, 100 *Vibrio hsp60* coding sequences were collected from the NCBI repository and blasted against the NCBI nucleotide database (nt file). Sequences were extracted using extract_hitseqs_from_sequences.pl and both accession numbers and their respective taxonomy were then extracted using list_basta_taxa.py provided by BASTA v1.3.2.3 (Kahlke and Ralph, 2019). The blast output was filtered to retain taxa assigned to the *Vibrio* genus using the filter_basta_fasta.py script also provided by BASTA (Kahlke and Ralph, 2019) and genes

assigned as *hsp60* and *groEL* were collected and added to our *Vibrio-hsp60* dataset. Both of these genes were chosen because they have previously been assigned as the same gene, but are annotated differently (Silvester et al., 2017). The *Vibrio-hsp60* data set was aligned with MAFFT (Katoh et al., 2017) using the einse –reorder option. The aligned untrimmed *hsp60* dataset was visualised using UGENE (Golosova et al., 2014) and highly conserved areas within the consensus sequence were chosen for primer construction. Primers were constructed using the Primer3Plus (Untergasser et al., 2007) software. The constructed degenerate primers were named Vib-hspF3-23 and Vib-hspR401-422 (Table 5.1), and their application resulted in the amplification of a 487 bp PCR product (Illumina adapters inclusive).

Table 5.1: Primers used in this study. Underlined sequences are Illumina sequencing adapters

Primer name	Sequence (5'-3')	Source
Vib-hspF3-23	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAACCCNATGGAYCTKAARCG	This study
Vib-hspR401-422	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCVATGATHARHAGHGRRCGNG	This study
16S 341F	AGAGTTTGATCMTGGCTCAG	(Herlemann et al., 2011)
16S 805R	GWATTACCGCGGCKGCTG	(Herlemann et al., 2011)
VF169	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGATAACYATTGGAAACGATG	(Yong et al., 2006)
Vib-680R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAATTCTACCCCCCTCTACAG	(Thompson et al., 2004)
Vib1-f	GGCGTAAAGCGCATGCAGGT	(Vezzulli et al., 2011)
Vib2-r	GAAATTCTACCCCCCTCTACAG	(Thompson et al., 2004)

To ensure that the *Vibrio* reference dataset was constructed with accurately assigned *Vibrio* taxa and not partial *hsp60* reads (which could possibly be assigned to the wrong taxa), we constructed a reference dataset using *hsp60* sequences taken from whole genomes. First, all of the currently available complete *Vibrio* genomes were collected from the NCBI repository (185 genomes) and a BLAST database was constructed using these genomes. *Vibrio hsp60* sequences were compared against this database and all hits at least 65 % similar to the query *hsp60* sequence and at least 400 base pairs long were extracted. BLAST hits were then visualised and trimmed to the primer locations in MEGA (version 7.0.26). To determine the coverage of *Vibrio* species in our dataset, we compared the taxa in this trimmed dataset against the listed *Vibrio* species in the NCBI taxonomy database. Where possible, *hsp60* sequences for missing *Vibrio* species were collected from incomplete whole genomes and added to the *Vibrio* reference dataset. This yielded a dataset comprising of 106 different *Vibrio* species incorporating 284 *hsp60* sequences. In some instances, *hsp60* was found in both *Vibrio* chromosomes. Where known, the second copy of the gene was named as 'group2'.

5.4.2 Mock Vibrio community preparation

Ten *Vibrio* species, spanning five clades (Sawabe et al., 2013; Turner et al., 2018) and incorporating species that are relevant to both human health (Daniels and Shafaie, 2000) and aquaculture diseases (Luna-González et al., 2002; Bruto et al., 2017; Go et al., 2017) were grown overnight in LB20 broth (per litre: 10 g tryptone, 5 g yeast extract, 20 g NaCl), with shaking at 28°C. Bacterial cells were enumerated using a Beckman CytoFLEX flow cytometer and cell counts were diluted to a standardised concentration across all strains. Three different mock *Vibrio* communities were prepared by mixing the 10 *Vibrio* species in different dilution ratios (Table 5.2). DNA

was then extracted from mock assemblages using the Qiagen DNeasy UltraClean Microbial Kit (catalogue: 12224-250) following the manufacturer's instructions.

Table 5.2: Composition of mock *Vibrio* communities generated in this study.

Vibrio species	Mock1 (%)	Mock2 (%)	Mock3 (%)
V. vulnificus	10	30	7.5
V. rotiferianus	10	5	7.5
V. sinaloensis	10	5	7.5
V. cholerae	10	30	7.5
V. campbellii	10	5	7.5
V. alginolyticus	10	5	7.5
V. diabolicus	10	5	7.5
V. harveyi	10	5	20
V. crassostreae	10	5	7.5
V. parahaemolyticus	10	5	20

5.4.3 Mock community PCR conditions and sequencing

DNA extracted from the mock bacterial communities was diluted to 10 ng μL^{-1} and used in a 50 μL PCR reaction volume as follows: 10 μL of 5 x Hi-Fi Buffer (Bioline), 5 μL of 10 mM dNTPs, 2 μL of high-fidelity Velocity polymerase (0.5 units μL^{-1}; Bioline), 2.5 μL of 10 μM forward primer (VF169 or Vib-hspF3-23), 2.5 μL of 10 μM reverse primer (Vib-680R or Vib-hspR401-422), 2 μL of DNA template (10 ng μL^{-1}), with the remaining volume made up with sterile water. The PCR mixture was then subjected to the following PCR conditions: one cycle of 98°C for 2 minutes, 30 cycles of 98°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, and a final extension time of 72°C for 10 minutes. PCR products were purified with a Bioline Isolate II PCR and Gel Kit (catalogue: BIO-52059) using the manufacturer's instructions. For 16S rRNA sequencing, extracted DNA were amplified with the following PCR conditions: 95°C for 3 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Mock bacterial community amplicons were characterised on the Illumina MiSeq platform (Ramaciotti Centre for Genomics; Sydney, NSW, Australia) using the manufacturers guidelines, using three primer sets (Table 5.1): the universal 16S rRNA primers 341F and 805R (Herlemann et al., 2011); a previously published *Vibrio*-specific 16S rRNA primer pair, VF169 (Yong et al., 2006) and Vib-680R (Thompson et al., 2004; Siboni et al., 2016); and the *Vibrio*-specific *hsp60* primer pair designed in this study, Vib-hspF3-23 and Vib-hspR401-422.

5.4.4 Mock community sequence analysis

Bacterial 16S rRNA and *hsp60* sequencing reads for the mock communities were processed as outlined in (Kahlke, 2018). Briefly, paired-end DNA sequences were joined using FLASH (Magoč and Salzberg, 2011) and subsequently trimmed using Mothur (Schloss et al., 2009a) (PARAMETERS: universal 16S - maxhomop=6, maxambig=0, qaverage=25, minlength=491, maxlength=501; *Vibrio*-specific 16S - maxhomop=6, maxambig=0, qaverage=25, minlength=533, maxlength=534; *hsp60* - maxhomop=6, maxambig=0, qaverage=25, minlength=420, maxlength=420). The resulting fragments were clustered at 97 % into operational taxonomic units (OTUs) and chimeric sequences were identified and removed using vsearch (Rognes et al., 2016). To assign taxonomy, QIIME (Caporaso et al., 2010) was used with the RDP classifier against either the Silva v128 database (for 16S rRNA analysed samples) or against our custom *Vibrio-hsp60* reference dataset. Sequences were then rarefied to the same depth to remove the effect of sampling effort upon analysis.

5.4.5 Seawater collection, 16S rRNA sequencing and data analysis

To test the newly designed *Vibrio*-specific *hsp60* sequencing assay on seawater samples, water was collected from Sydney Harbour (33.839S, 151.254E) in the Austral summer. Seawater was filtered in triplicate through 0.22 μm membranes and the filters immediately snap frozen in liquid nitrogen. Microbial DNA was subsequently extracted from filters using a Qiagen DNeasy PowerWater kit (catalogue: 14900-100-NF) and sent to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for 16S rRNA (341F and 805R) sequencing on the Illumina MiSeq platform.

Raw 16S rRNA demultiplexed paired-end DNA sequences were joined using Flash (Magoč and Salzberg, 2011) and trimmed with Mothur (Schloss et al., 2009a) (PARAMETERS: maxhomop=6, maxambig=0, qaverage=25, minlength=441, maxlength=466). Fragments were then clustered into OTUs at 97 % and chimeric sequences removed using vsearch (Rognes et al., 2016). QIIME and the RDP classifier were then used to assign taxonomy against the Silva v128 database. Sequences were then rarefied.

5.4.6 Seawater hsp60 PCR conditions, sequencing and data analysis

DNA from seawater were amplified using the Vib-hspF3-23 and Vib-hspR401-422 primer pair with the Illumina adapters added to the primers (Table 5.1). The 30 μL PCR reaction mixture was as follows: 6 μL of 5 x Hi-Fi Buffer (Bioline), 3 μL of 10 mM dNTPs, 0.5 μL of high-fidelity Velocity polymerase (2 units μL^{-1}; Bioline), 1.5 μL of 20 μM forward primer, 1.5 μL of 20 μM reverse primer, 3.5 μL of template DNA, and the remainder (14 μL) made up with sterile water. The mixture was used with the following touchdown PCR conditions: one cycle of 98°C for 2 minutes, 5 cycles of 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, 21 cycles of 98°C for 30 seconds, 60°C for 30 seconds with a reduction of 0.5°C per cycle (60°C to 50°C), and 72°C for 45 seconds, 14 cycles of 98°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, and a final extension time of 72°C for 10 minutes. Amplicons were then purified by the Ramaciotti Centre for Genomics, and characterised on the Illumina MiSeq platform using the manufacturers guidelines.

As the *Vibrio*-specific *hsp60* primer pair were found to in some scenarios non-specifically amplify other taxa, a further cleaning step was added to the data analysis. In the first instance, pair-ended sequences were joined using

FLASH (Magoč and Salzberg, 2011) and trimmed using mothur (Schloss et al., 2009a) (PARAMETERS: maxhomop=5, maxambig=0, qaverage=25, minlength=420, maxlength=420). These fragments were then clustered at 97% into operational taxonomic units (OTUs) and chimeric sequences were identified and removed using vsearch (Rognes et al., 2016) against the *Vibrio-hsp60* reference dataset. To remove reads not belonging to the *Vibrio* genera, a BLAST database was constructed using the cleaned OTU fasta file after removing chimeras. The *Vibrio-hsp60* reference dataset was then blasted against the cleaned OTU fasta file, and OTUs that were 90 % similar to sequences in the *Vibrio-hsp60* reference dataset, and over 400 bp in length, were retained. The best BLAST hit for each OTU was then extracted, therefore removing the possibility of retaining multiple BLAST hits for each OTU. This fasta file was then used to assign taxonomy against with the RDP classifier. Due to the large spread of reads per sample, data were not rarefied, reads were normalised to the number of reads per sample to produce the relative abundance of each taxa for each sample.

5.4.7 Quantitative PCR (qPCR)

To provide an indication of *Vibrio* abundance in each sample, a quantitative PCR (qPCR) assay was used to quantify the number of *Vibrio*-specific 16S rRNA gene copies in each sample using the *Vibrio*-specific 16S rRNA gene primers Vib1-f and Vib2-r (Table 5.1) (Thompson et al., 2004; Vezzulli et al., 2011; Siboni et al., 2016). qPCR was performed using an epMotion 5075l Automated Liquid Handling System on a Bio-Rad CFX384 Touch Real-Time PCR Detection System with a seven-point calibration curve and a negative control. The calibration curve was created from 10-fold dilutions of a known quantity of amplicon DNA, measured by a Qubit device. All sample analyses were performed with three technical replicates, using the following reaction mixture: 2.5 µL iTaq Universal SYBR Green supermix, 0.4 mM of

each forward and reverse primer, 1 μL of template DNA (50ng μL^{-1}), and the remainder made up with water. The qPCR cycling conditions were as follows: 95°C for 3 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The resulting data were normalised to millilitres of collected water. A coefficient of variation (CV) was then calculated for the technical triplicates, and where necessary, samples with CV > 1 % had a replicate removed from the analysis. A melting curve was added to the end of every run to confirm the presence of a single PCR product.

5.4.8 Laboratory-induced oyster mortality event

The newly designed *Vibrio*-specific *hsp60* primer set was applied to examine patterns in *Vibrio* community diversity during a previously described laboratory-induced Pacific Oyster mortality event, where *Vibrio* species had previously been implicated as the cause of oyster mortality during a simulated marine heatwave (Green et al., 2019).

Briefly, triploid Pacific oyster (*C. gigas*) spat were collected from Port Stephens, New South Wales, Australia, prior to a forecasted marine heat wave. Spat were held in two different temperature conditions (low 20°C ± 1°C and high 25°C ± 1°C) with and without antibiotics (100 units/ml of penicillin and 0.1 mg/ml of streptomycin) and monitored for six days. Spat were placed in sterilised glass tanks, and UV and 5 μm filter sterilised seawater were added and replaced daily. Triplicate oyster spat were sampled on days 0, 3, 4, 5, and 6, and dead oysters were removed and frozen at -80 °C prior to processing. Cumulative mortality was 77.4 ± 10.7 % and 3.4 ± 5.9 % for the high and low temperature treatment respectively, with antibiotics in the high temperature treatment reducing the cumulative mortality to 4.3 ± 3.7 %. Mortalities were greatest between days three to five. For the purposes of this study, DNA extracted from spat exposed to the high

and low temperature treatments from each sampling point, were amplified with the *Vibrio*-specific *hsp60* primer pair (Table 5.1). *Vibrio* dynamics were previously characterised within oyster tissues using a combination of culture-based approaches, quantitative PCR and 16S rRNA amplicon sequencing (Green et al., 2019). Oyster DNA were subject to *hsp60* PCR amplification, sequencing and data analysis, and qPCR, as described above for the seawater samples, with the exception of DNA being diluted to 50 ng μL^{-1} for the PCR conditions.

5.4.9 Statistical analyses

Comparisons of community compositions were performed with a non-metric multidimensional scaling analysis (nMDS) with a Bray-Curtis dissimilarity index, using data normalised to the number of reads per sample, reads less than 1 % relative abundance removed and then transformed (square root). Patterns observed in the nMDS analysis were statistically tested with a one-way PERMANOVA with 9999 permutations. To examine the similarity between each characterised community to the mock community, similarity indices were calculated with a Bray-Curtis dissimilarity index using data that was filtered, transformed and with reads assigned to the second chromosome (group2) combined with their respective assigned species. To examine the contribution of individual *Vibrio* species to community dissimilarity, a SIMPER analysis with untransformed data was used with a Bray-Curtis dissimilarity index. To determine the relationship between *Vibrio*-specific 16S rRNA gene copies and the yield of *hsp60* reads from the QIIME analysis, an ordinary least squares linear regression was used. Spearman's rank correlation was used to examine relationships between *Vibrio* species (summarised at the species level) and oyster mortality. All statistical comparisons were performed in the PAST statistical environment (Hammer et al., 2001).

5.5 Results and Discussion

5.5.1 Comparison of Vibrio mock community characterisation using 16S rRNA and hsp60

Mock *Vibrio* communities consisting of 10 different *Vibrio* species were characterised using the 16S rRNA, *Vibrio*-specific 16S rRNA and *Vibrio*-specific *hsp60* primer pairs followed by Illumina MiSeq sequencing of the amplicons. When examined on a non-metric multidimensional scaling analysis (nMDS), the *Vibrio* community structure defined by the *Vibrio*-specific *hsp60* assay clustered closer to the true mock community, with the true mock community sitting within the 95 % ellipses for each *Vibrio*-specific *hsp60* characterised community (Figure 5.1A-C). Comparatively, the compositions of the 16S rRNA, *Vibrio*-specific 16S rRNA, and *Vibrio*-specific *hsp60* characterised communities were on average 16, 25, and 77 % similar to the true mock community, respectively (Figure 5.1D).

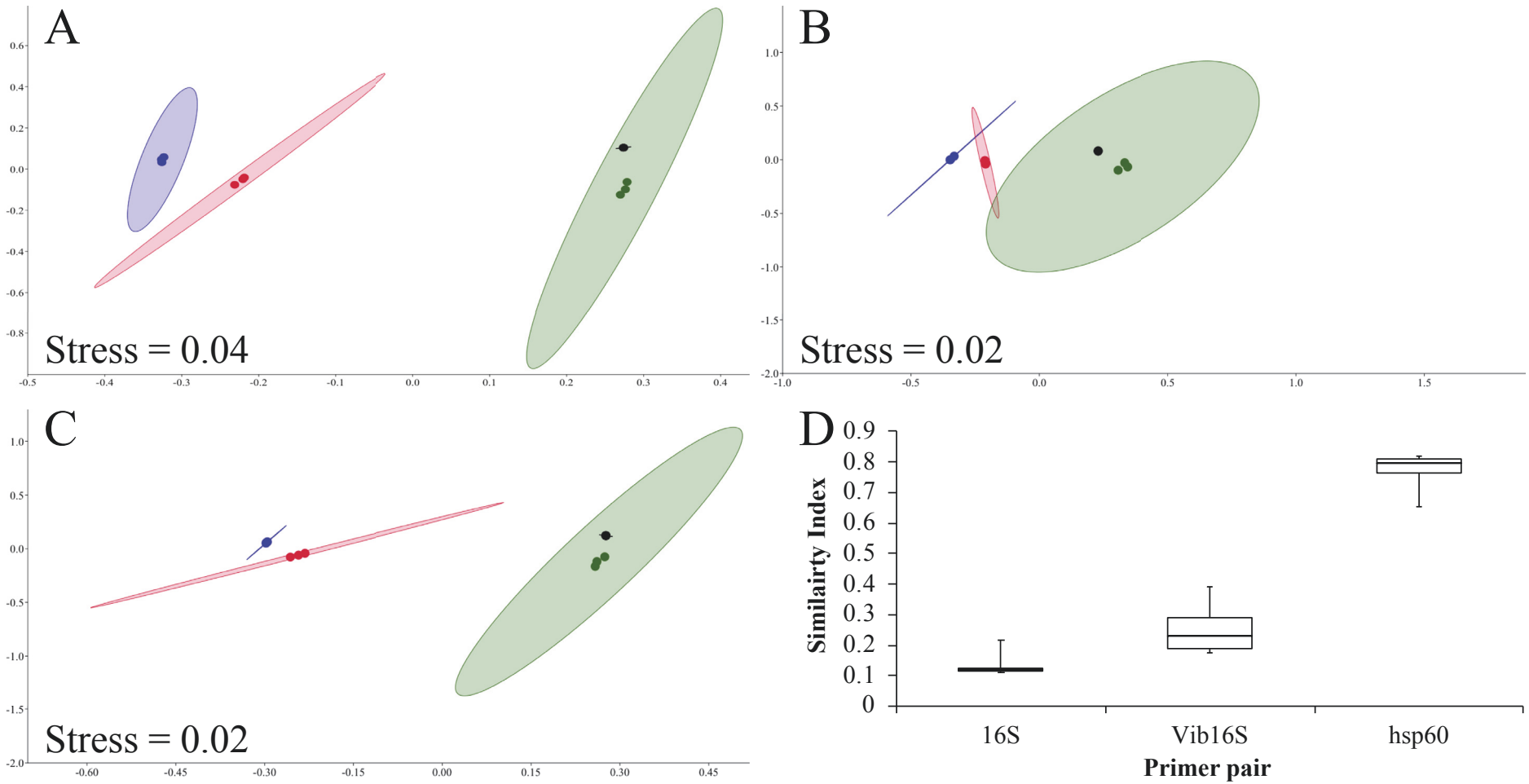


Figure 5.1: nMDS analysis of the 16S rRNA (blue dots), *Vibrio*-specific 16S rRNA (red dots) and *Vibrio*-specific *hsp60* (green dots) characterised mock communities, and the true mock community (black dots). Mock communities 1, 2 and 3 are panes A, B and C respectively. 95 % ellipses are shown. Panel D: Box and whisker plot of Bray-Curtis similarity comparisons of community composition compared to the true mock communities. Data for all three mock communities is combined. For species assigned across two taxonomic assignments (e.g. group 2), they were combined with their respective species for panel D.

The *Vibrio* community data derived from the traditional V3-V4 16S rRNA primer pair was poorly characterised beyond the genus level, with only one *Vibrio* species used in the mock community correctly identified (Figure 5.2). On average, reads were not defined beyond the *Vibrio* genus level 90, 74 and 89 % of the time in mock communities 1, 2 and 3 respectively. Of the sequences that were assigned beyond the *Vibrio* genus level, they were only assigned to *V. cholerae* and *V. azureus*. Notably, *V. azureus* was not part of the mock communities indicating not only imprecise, but incorrect taxonomic classification. *V. azureus* is closely related to the *V. harveyi* clade, for which it was probably incorrectly attributed (Yoshizawa et al., 2009). Reads assigned to *V. cholerae* were correctly assigned but were marginally under-represented when compared to the mock community (6 - 23 % for 16S rRNA; 7.5 - 30 % for the mock communities).

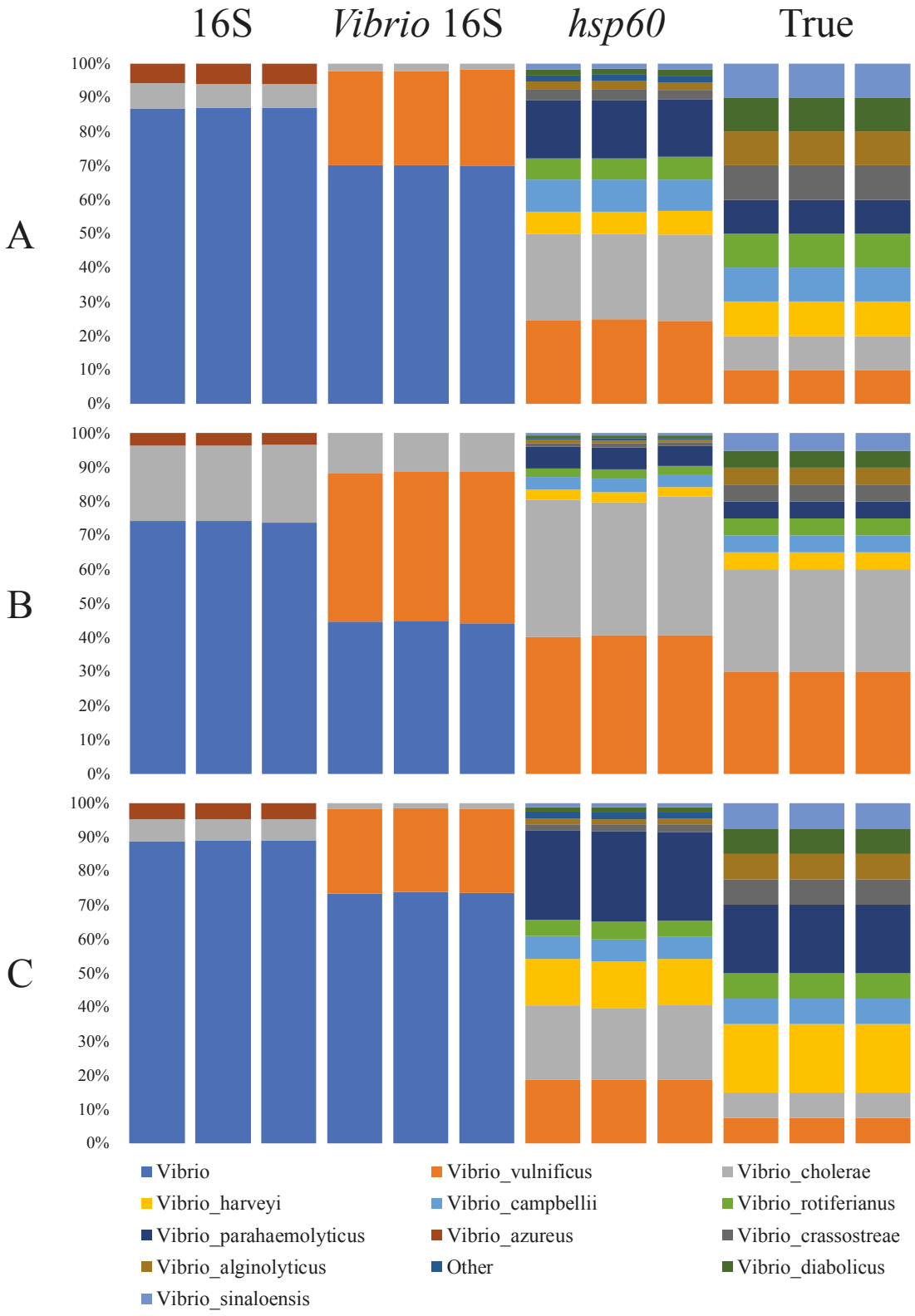


Figure 5.2: Comparison of amplicon sequenced phylogenetic markers for the *Vibrio* mock communities. Mock communities 1, 2, and 3 are A, B, and C respectively. Communities were characterised using 16S rRNA V3-V4 (Herlemann et al., 2011), *Vibrio*-specific 16S rRNA (Thompson et al., 2004; Yong et al., 2006), and *Vibrio*-specific *hsp60* primer pairs. The true mock community composition is also shown. Displayed data is relative abundance summarised at the species level. For reads assigned to the second chromosome (group2), they were combined with their respective species. Reads representing less than 1 % of the relative abundance were removed.

Similar to the 16S rRNA characterised community composition, the data derived from the *Vibrio*-specific 16S rRNA sequencing assay was only able to identify two *Vibrio* species used in the mock community, with the majority of the reads not resolved beyond the genus level. Although correctly identified as *Vibrio*, the majority of sequences could not be assigned to the species level 70, 45 and 74 % of the time in mock communities 1, 2 and 3 respectively, an improvement to those observed for the 16S rRNA characterised communities. For the remainder of the reads assigned beyond the genus level, they were correctly assigned to *V. vulnificus* and *V. cholerae*. Reads assigned to *V. vulnificus* were over-represented when compared to the mock community (25-44 % for *Vibrio*-specific 16S rRNA; 7.5-30 % for the mock communities), while reads assigned to *V. cholerae* were under-represented (2-12 % for *Vibrio*-specific 16S rRNA; 7.5-30 % for the mock communities).

Relative to both of the 16S rRNA based sequencing assays, the *Vibrio*-specific *hsp60* primer set identified the greatest number of species in the *Vibrio* community, with all of the species present in the mock community correctly identified by this assay. While all of the species were correctly identified, differences in the relative abundance of each species was observed when compared to the true mock community (Table 5.3). *V. campbellii* was the best represented species with each *Vibrio*-specific *hsp60* characterised mock community only showing a 1 % difference to the true mock community, while *V. sinaloensis* was the most under-represented species with differences of 4-8 % for the *Vibrio*-specific *hsp60* characterised communities. For *V. vulnificus* and *V. cholerae*, the only two correctly identified species in the 16S rRNA and *Vibrio*-specific 16S rRNA characterised communities, the *Vibrio*-specific *hsp60* assay provided marginally better representation for *V. vulnificus* (over-representation of 10-

13 % compared to the mock community) compared to the *Vibrio*-specific 16S rRNA assay (over-representation of 14-18 %) and 16S rRNA assay (not identified). For *V. cholerae*, this species was under-represented in both the 16S rRNA (1-8 %) and *Vibrio*-specific 16S rRNA assays (6-20 %) compared to an over-representation in the *Vibrio*-specific *hsp60* assay (9-14 %). The exaggeration of *V. vulnificus* and *V. cholerae* could possibly be due to a greater *hsp60* primer affinity to these species, or the presence of two copies of *hsp60* in the genomes of these bacteria (one copy was identified in each chromosome for these two species).

Notably, the *Vibrio*-specific *hsp60* sequencing assay also distinguished members of the *V. harveyi* clade, a tight phylogenetic group within the *Vibrio* genus (Sawabe et al., 2013; Urbanczyk et al., 2013), which has previously had numerous incorrect taxonomic assignments to species within this clade due to their close 16S rRNA genetic similarity (Lin et al., 2010; Sawabe et al., 2013; Urbanczyk et al., 2013). This clade includes *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *V. campbellii*, *V. diabolocus* and *V. rotiferianus*, all of which were identified with the *Vibrio*-specific *hsp60* sequencing assay (Sawabe et al., 2013; Turner et al., 2018). Many of these species are important pathogens (Daniels and Shafaie, 2000; Luna-González et al., 2002; Go et al., 2017) and therefore accurately identifying their presence in environmental samples is an important requisite of a *Vibrio* specific assay of this type.

Table 5.3: Relative abundance comparisons between the *Vibrio*-specific *hsp60* characterised mock communities and the true mock communities. Displayed 1 % filtered relative abundance is averaged across three replicates and in those cases where reads were assigned to the second chromosome (group 2), they were combined with their respective species. Relative abundance differences are also shown. 1, 2 and 3 represent mock communities 1, 2 and 3 respectively.

Taxa	hsp60_1	True_1	Difference_1	hsp60_2	True_2	Difference_2	hsp60_3	True_3	Difference_3
V. vulnificus	23.4	10	13.4	39.8	30	9.8	17.9	7.5	10.4
V. cholerae	23.9	10	13.9	39.2	30	9.2	20.9	7.5	13.4
V. parahaemolyticus	16.2	10	6.2	6.1	5	1.1	25.3	20	5.3
V. harveyi	6.4	10	-3.6	2.8	5	-2.2	13.1	20	-6.9
V. campbellii	8.8	10	-1.2	3.8	5	-1.2	6.2	7.5	-1.3
V. rotiferianus	6.0	10	-4.0	2.6	5	-2.4	4.7	7.5	-2.8
V. alginolyticus	7.4	10	-2.6	2.7	5	-2.3	5.7	7.5	-1.8
V. crassostreae	2.9	10	-7.1	1.1	5	-3.9	1.9	7.5	-5.6
Other	1.7	0	1.7	0.6	0	0.6	1.8	0	1.8
V. diabolicus	1.8	10	-8.2	0.8	5	-4.2	1.4	7.5	-6.1
V. sinaloensis	1.6	10	-8.4	0.6	5	-4.4	1.2	7.5	-6.3

Previous attempts to perform *hsp60* amplicon sequencing have used universal *hsp60* primers and filtered the data for the assigned *Vibrio* sequences (Jesser and Noble, 2018). Only 0.5 % of the total *hsp60* data were assigned to *Vibrio* species in the previous study, compared to retaining 21.1 % of the data for the *Vibrio*-specific *hsp60* assay produced in this study. Further, the remaining 0.5 % included a significant number of unassigned *Vibrio* species (Jesser and Noble, 2018), attributable to poor *Vibrio* species representation in the *cpn60* database (Hill et al., 2004; Jesser and Noble, 2018). The *Vibrio* reference dataset produced in this study encompasses 106 different *Vibrio* species compared to only 63 unique species in the *cpn60* database (accessed August 2019). Therefore, the assay produced in this study delivered greater data yield compared to using universal *hsp60* primers and included more *Vibrio* species in the reference dataset.

5.5.2 Vibrio diversity in seawater

After confirming the utility of the *Vibrio*-specific *hsp60* sequencing assay using mock communities, this assay was used to characterise *Vibrio* diversity in seawater samples collected from Sydney Harbour, with the measured community composition compared to that derived from traditional 16S rRNA sequencing (Figure 5.3). Reads assigned to the *Vibrio* genus or *Vibrio* species only made up 0.13-0.17 % of the total bacterial community using 16S rRNA sequencing, with the majority (59-77 % relative abundance) of these reads not resolved beyond the *Vibrio* genus-level. In contrast, the proportion of the community assigned to the *Vibrio* genus when using the Vibrio-specific *hsp60* sequencing assay was only 1.4-1.7 %. Ten different *Vibrio* species were identified within the seawater samples using the *Vibrio*-specific *hsp60* sequencing assay, most of which were lowly abundant (1-4 % relative abundance) except for *V. azureus* (58-71 %) and *V. mediterranei* (10-29 %). In contrast, only three *Vibrio* species were identified using the

16S rRNA assay. Both assays identified the presence *of V. mediterranei,* with similar levels of relative abundance (16S rRNA: 19-34 %; *hsp60*: 10-29 %). The unique co-occurrence of *V. azureus* and *V. mediterranei* in seawater has been observed in a previous study (Amin et al., 2016) and may explain the co-dominance of these species in these *Vibrio* seawater communities.

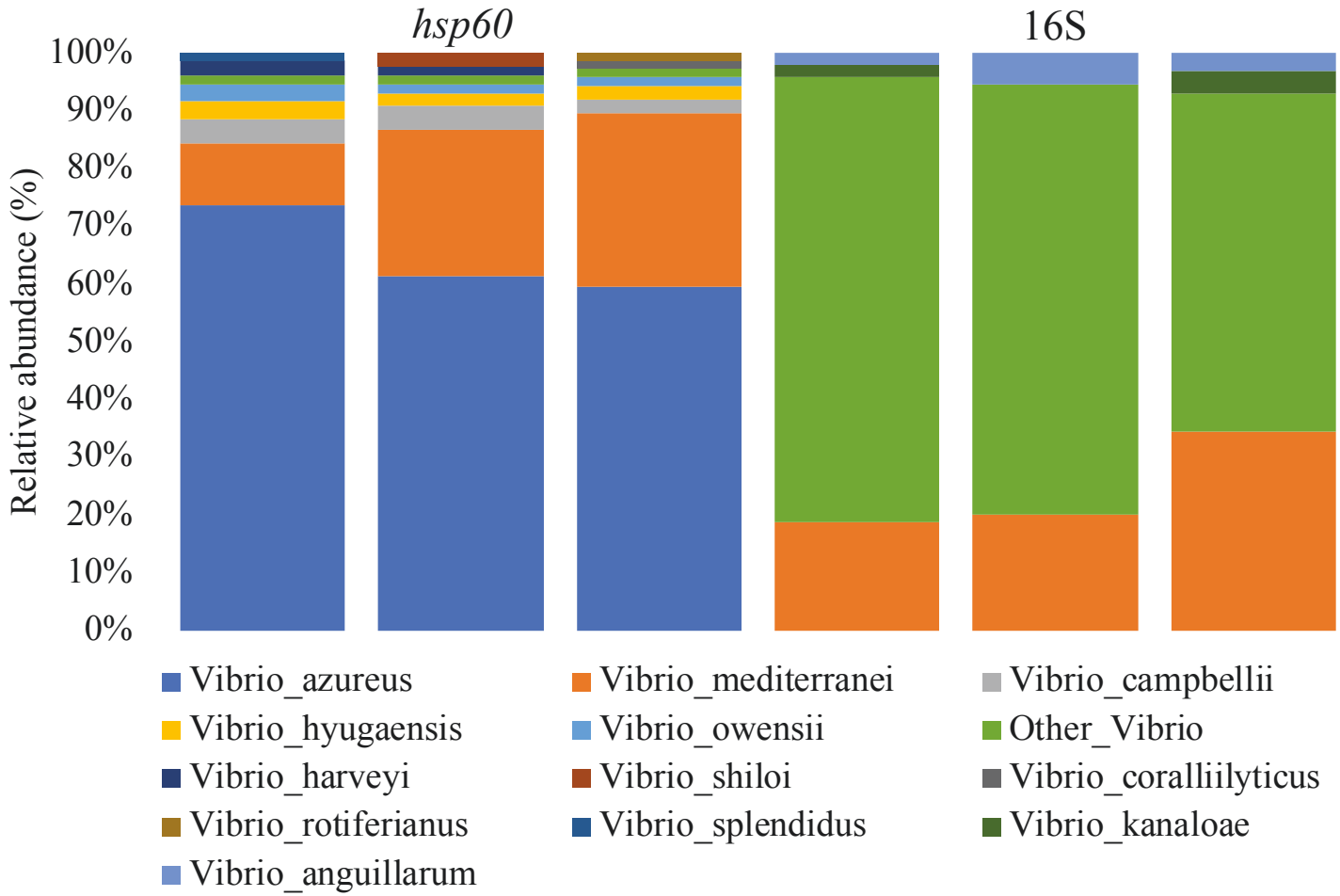


Figure 5.3: *Vibrio* diversity in seawater from Sydney Harbour. DNA were characterised with the *Vibrio*-specific *hsp60* and 16S rRNA V3-V4 (Watermann et al., 2008) primer sets. Displayed data is relative abundance summarised at the species level.

5.5.3 Vibrio abundance determines assay efficacy

To determine assay efficiency, both seawater and oyster samples were used. As expected, samples with the greatest abundance of *Vibrio,* as determined using qPCR targeting *Vibrio* 16S rRNA gene copies, had the greatest number of *hsp60* reads (Supplementary Figure 5.1), with a significant relationship observed between *Vibrio* 16S rRNA gene copies and *hsp60* reads ($R^2 = 0.87$; $p = 0.0001$) (Figure 5.4). It is possible that the low number of *hsp60* reads in samples with low *Vibrio* biomass was due to non-specific amplification of *hsp60* sequences associated with other bacterial genera. However, when significant levels of *Vibrio* are present within a sample, this assay delivers substantial capacity to probe the diversity of the community.

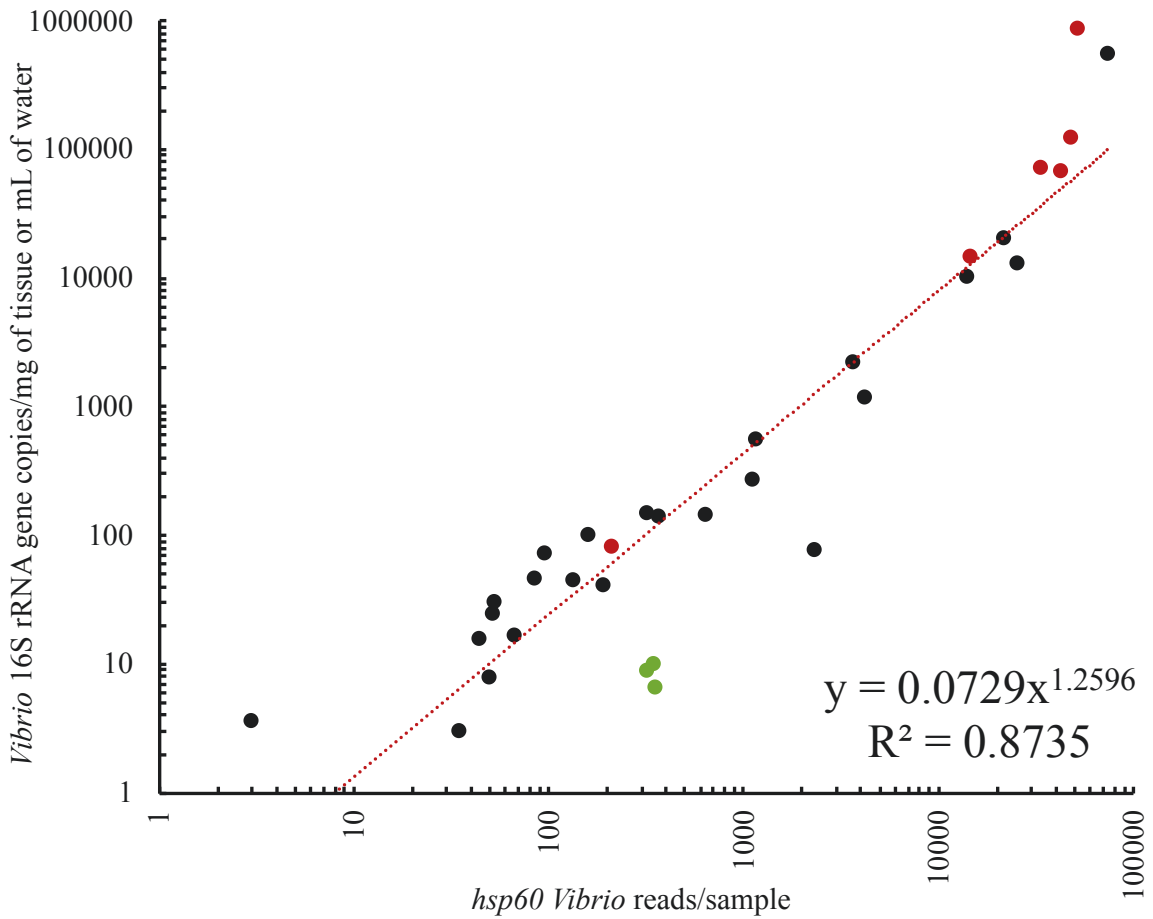


Figure 5.4: Ordinary least squares linear regression of *Vibrio* 16S rRNA gene copies and *hsp60* reads per sample. Black dots are oyster samples, red dots are oyster mortality samples and green dots are seawater samples. Both axes are logarithmic in scale.

5.5.4 *Vibrio diversity during a laboratory induced oyster mortality event*

After confirming the utility of the *Vibrio*-specific *hsp60* assay to track *Vibrio* community dynamics with high fidelity using a mock community and successfully applying it to characterise *Vibrio* diversity within natural seawater samples, it was next used to examine patterns in *Vibrio* diversity during a laboratory-induced oyster mortality. During this simulated heatwave event described in detail in Green et al. (2019), significant levels of oyster mortality were observed in oysters exposed to an increase in water temperature to 25°C (77.4 ± 10.7 %), relative to oysters maintained at ambient temperature levels at 20°C (3.4 ± 5.9 %). The *Vibrio*-specific *hsp60* assay was applied on samples derived from this study, because previous analyses suggested that *Vibrio* were implicated and overly abundant in this mortality event (Green et al., 2019).

Using the *Vibrio*-specific *hsp60* sequencing assay, the *Vibrio* community composition associated with Pacific oysters were significantly different in accordance with differences in temperature ($F = 6.5$, $p = 0.0005$; Supplementary Figure 5.2) and oyster mortality ($F = 14.8$, $p = 0.0003$ versus low temperature; $F = 4.4$, $p = 0.013$ versus high temperature). The 'baseline' *Vibrio* community (Figure 5.5) on the first day of the experiment (day zero), four days prior to significant mortalities, was distributed across nine different species, with *V. brasiliensis*, *V. chagasii*, *V. fortis*, and *V. harveyi* representing the dominant members of the *Vibrio* community with average relative abundances of 9, 20, 11, and 35 % respectively.

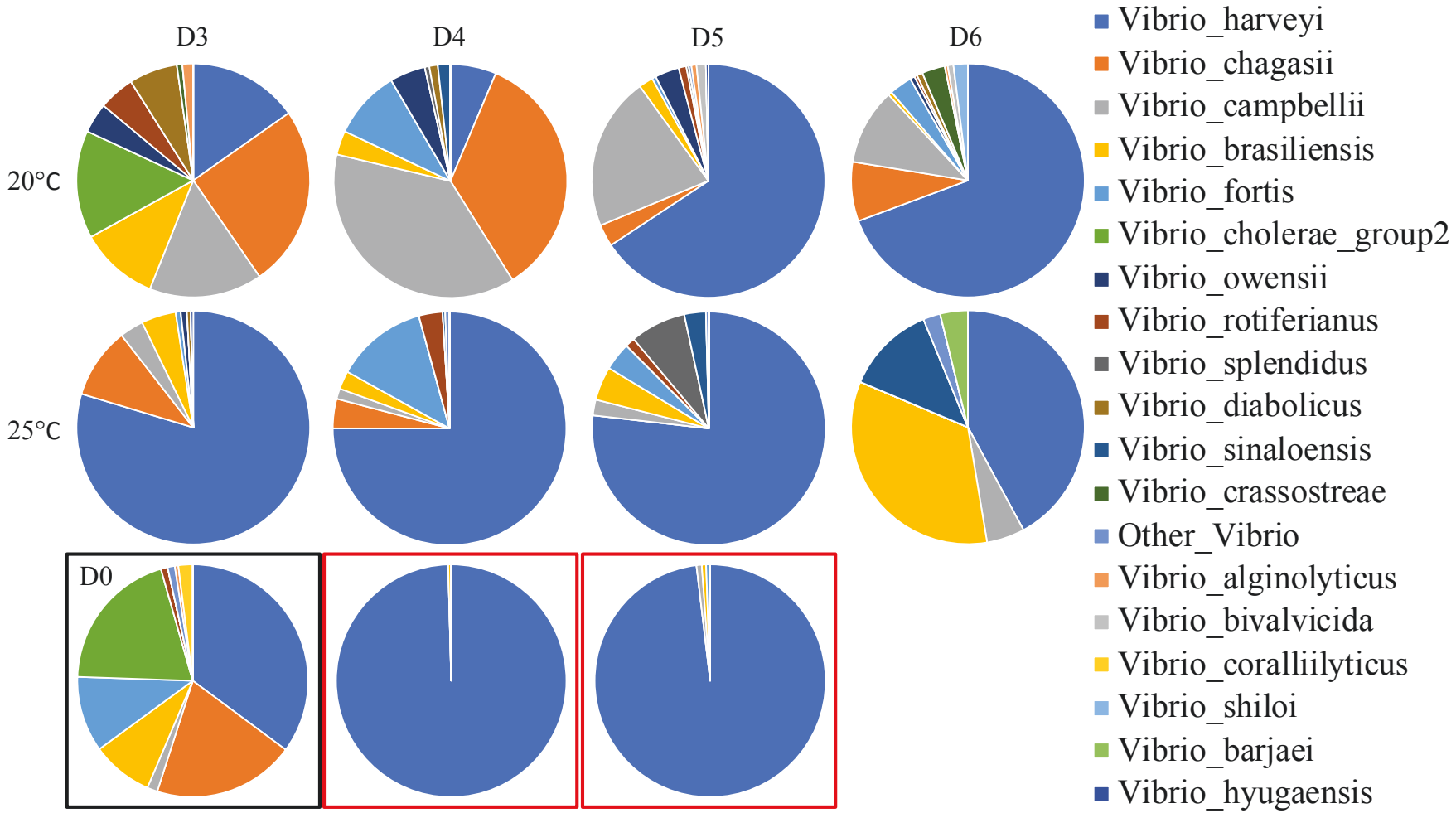


Figure 5.5: *Vibrio* community of *C. gigas* spat across six days and two temperature treatments. D0, D3, D4, D5, and D6 correspond to sampling days zero through to six. Communities are averaged across three biological replicates and summarised at the species level. Communities in a black box are day zero. Communities in red boxes are dead *C. gigas* spat from the high (25°C) temperature treatment, taken on days four and five respectively. Reads representing less than 1% of the relative abundance were removed.

When comparing temperature treatments, the *Vibrio* communities were on average 56 % dissimilar to each other. In the low temperature treatment, *V. campbellii* and *V. chagasii* were the most prominent members, contributing 18 and 15 % to the community dissimilarity (21 and 17.6 % average relative abundance respectively; Supplementary Table 5.1) and were both negatively correlated to temperature (r_s = -0.4, p = 0.04; r_s = -0.53, p = 0.008, respectively) and mortality (r_s = -0.45, p = 0.02; r_s = -0.52, p = 0.007, respectively. While, *V. harveyi* dominated the *Vibrio* community in the high temperature treatments contributing 37 % to the community dissimilarity between temperature treatments and was positively correlated to temperature (r_s = 0.52, p = 0.011) and mortality (r_s = 0.55, p = 0.006). On days three, four, and five, *V. harveyi* comprised 73-75 % of the whole community, followed by a decrease in relative abundance (41 %) on day six. This pattern is consistent with the results of a *V. harveyi* specific qPCR assay performed on these samples in a previous study, where a significant increase in copies of the *V. harveyi* gyrase B gene was observed on days three, four and five, followed by a decrease on day six (Green et al., 2019).

Notably, a sharp increase in the relative abundance of *V. harveyi* was also observed in the low temperature treatment on days five (6 % on day four to 65 %) and six (68 %), which was again consistent with qPCR data (Green et al., 2019). Dead oyster samples collected on days four and five from the high temperature treatment were also completely dominated by *V. harveyi*, which represented 97 % and 96 % of the *Vibrio* community respectively. Low levels of oyster mortality (2%) were observed in the low temperature treatment on day six (Green et al., 2019), which notably corresponded with an increase in the relative abundance of *V. harveyi* on the preceding day (6 to 65 % from days four to five). *V. harveyi* was previously implicated as the causative agent behind this mortality event (Green et al., 2019) and a

previous study implicated *V. harveyi* as a causative agent for an unknown mass mortality outbreak from the same region the oysters were sourced from (Port Stephens) (Go et al., 2017). The data derived from the *Vibrio*-specific *hsp60* sequencing approach was able to unambiguously pinpoint the putative pathogen that increased in abundance prior to disease onset, as evidenced by previous culturing studies (Go et al., 2017; Green et al., 2019).

Temperature was strongly correlated to mortality ($r_s = 0.87$, $p = 0.0001$) and may have provided a selective advantage for *V. harveyi* allowing for an increase in the relative abundance of this species, effectively replacing the putative commensal *Vibrio* species (Lemire et al., 2015) and/or temperature may have acted as an immunosuppressant in the oysters allowing for a shift in the *Vibrio* community preceding disease (Lokmer and Wegner, 2015). Interestingly, the oysters on day six in the high temperature treatment had a decreased number of reads assigned to *V. harveyi* relative to the preceding days (75 to 45 % from days five to six). A possible explanation for this pattern is that a sub-population of surviving oysters exhibited higher tolerance to the elevated temperature conditions, avoided colonisation by *V. harveyi* and survived. These results indicate that temperature stressed oysters undergo a substantial shift in the composition of their *Vibrio* community, involving a dramatic increase in the relative abundance of *V. harveyi*, which precedes oyster mortality. Both the occurrence of elevated levels of the *V. harveyi* in oysters before and during mortality and the very high levels of *V. harveyi* in freshly deceased oysters further implicate this species in oyster mortality events, in agreement with previous studies (Saulnier et al., 2010; Segarra et al., 2010; Jenkins et al., 2013; Le Roux et al., 2016; Go et al., 2017).

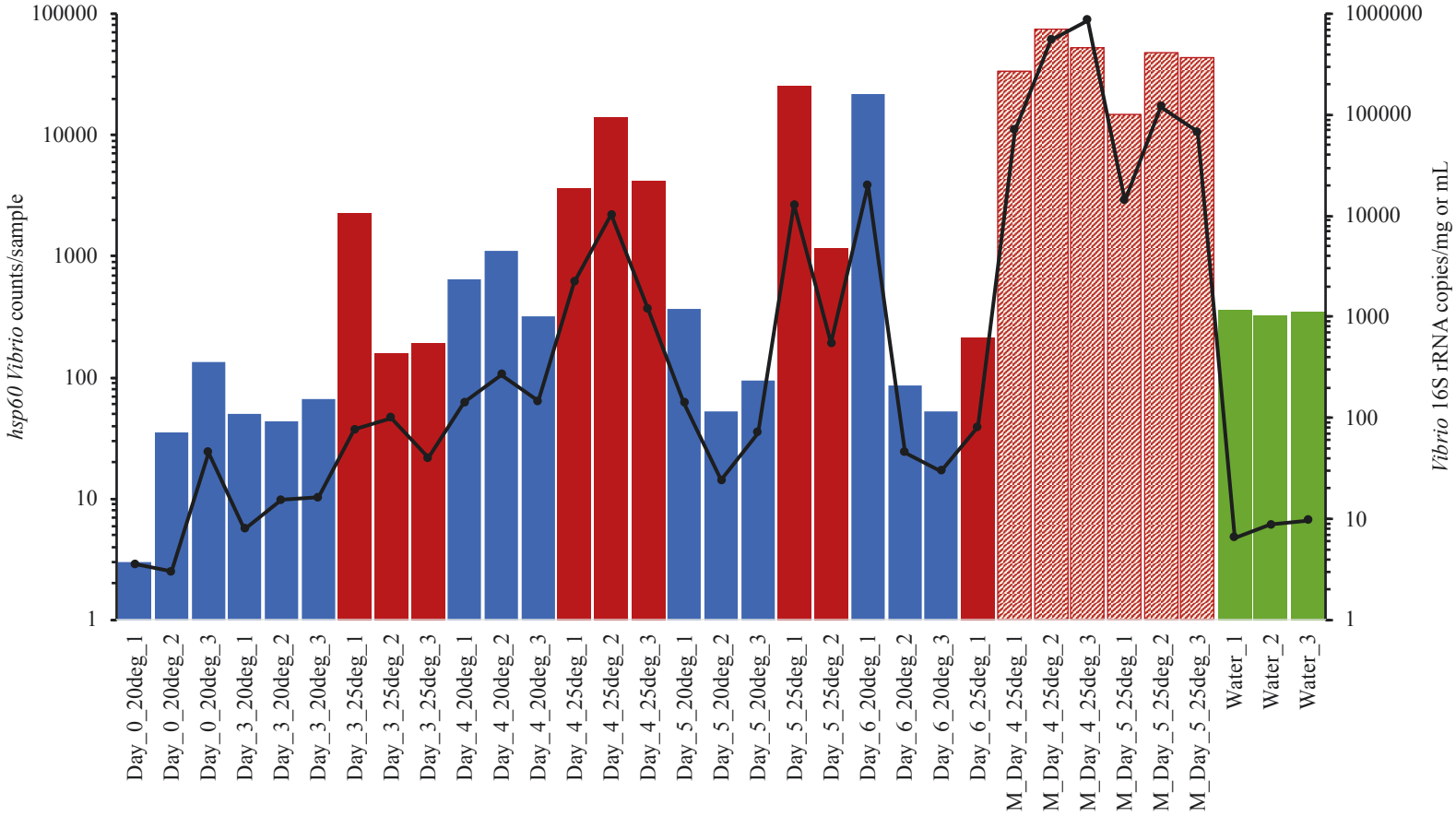
5.6 Conclusion

Most standard approaches for examining *Vibrio* diversity are constrained by poor taxonomic resolution beyond the genus level. This is often a significant limitation because *Vibrio* species are often implicated in disease events among both natural populations of marine organisms (Kushmaro et al., 2001; Austin and Zhang, 2006; Rubio-Portillo et al., 2014) and commercially important aquaculture species (Goarant et al., 2000; Becker et al., 2004; Frans et al., 2011; Geng et al., 2014; Vezzulli et al., 2015). Here, a *Vibrio*-specific *hsp60* sequencing assay was created using primers tailored to *Vibrio*-specific *hsp60* and used in combination with a custom-built *Vibrio* reference dataset including 106 *Vibrio* species. The sequencing assay was able to successfully identify every *Vibrio* species included within a mock community constructed with known dilutions of different *Vibrio* species. Despite an exaggeration in the relative abundance of some species, the *Vibrio*-specific *hsp60* sequencing assay provided greatly superior taxonomic resolution when compared to conventional 16S rRNA sequencing methods. The *Vibrio*-specific *hsp60* sequencing assay was subsequently successfully applied to seawater samples providing better discrimination of *Vibrio* diversity compared to 16S rRNA amplicon sequencing approaches, highlighting its utility in seawater. Next, the sequencing assay was able to unambiguously identify the *Vibrio* species that increased in abundance during an oyster mortality event, pinpointing a putative pathogen involved in the deaths of oysters following a simulated marine heatwave. This *Vibrio*-specific *hsp60* sequencing assay offers the potential for high throughput characterisation of *Vibrio* diversity while retaining a highly specific degree of taxonomic resolution in environmental samples, important for dissecting species level community dynamics and their relationship with the environment or disease.

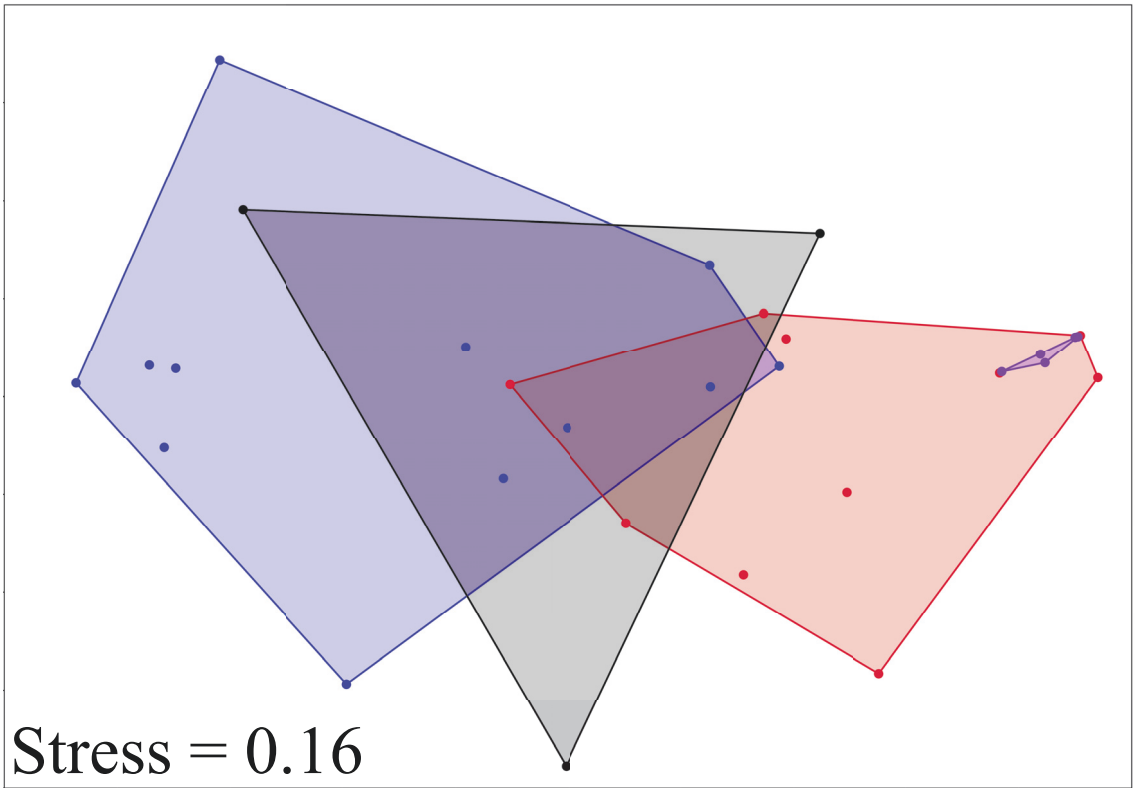
5.7 Acknowledgements

This research was supported by an Australian Research Council Linkage Project (LP160101785) to JRS and ML; 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.

5.8 Supplementary Information



Supplementary Figure 5.1: Comparison of *hsp60* assigned reads of each sample to *Vibrio*-specific 16S rRNA qPCR. Blue and red columns are samples from the low (20°C) and high (25°C) temperature treatment respectively, while green columns are water samples. The *hsp60* reads (primary x-axis; columns) were the highest in mortality samples (red hatched), followed by the high temperature treatment. The number of *Vibrio* 16S rRNA gene copies (secondary x-axis; black line) was elevated in accordance with the number of *hsp60* reads. Both axes are logarithmic in scale.



Supplementary Figure 5.2: Non-metric multidimensional scaling analysis of oyster *Vibrio* community composition. Blue and red dots are the low and high temperature treatments respectively. Black dots are the first day of sampling (day zero) and purple dots are the dead oyster samples.

Supplementary Table 5.1: SIMPER analysis of *Vibrio* communities between temperature treatments. Dissimilarity contribution is provided with the average of each species in each treatment.

Vibrio species	Dissimilarity contribution (%)	Low temperature average (%)	High temperature average (%)
V. harveyi	37.07	38.8	72.5
V. campbellii	17.73	21	2.57
V. chagasii	14.91	17.6	4.12
V. brasiliensis	6.91	4.17	6.88
V. fortis	5.282	3.24	5.08

Supplementary Table 5.2: Spearman's correlation between oyster mortality, temperature and *Vibrio* species. Data is presented as r_s value, p-value. NS is not significant.

Variable	Mortality	Temperature
V. harveyi	0.55, 0.0057	0.52, 0.0011
V. chagasii	-0.52, 0.0065	-0.53, 0.008
V. campbellii	-0.45, 0.025	-0.41, 0.042
V. owensii	-0.55, 0.0044	-0.51, 0.014
V. sinaloensis	NS	0.41, 0.046
Temperature	0.87, 0.0001	N/A

Chapter Six

General discussion and future directions

The overarching goal of this thesis was to provide an improved understanding of oyster health and disease using a microbiome approach. In achieving this goal, this thesis has contributed new knowledge and new tools to the wider scientific community and oyster industry. First, a comprehensive literature review highlighting the known links between oyster disease, environment and the microbiome was produced, and identified knowledge gaps and future research directions particularly with regard to the potential role(s) of the oyster microbiome (Chapter One – published in Marine Environmental Research). Second, the microbiomes of *Crassostrea gigas* affected by a summer mortality disease outbreak in Port Stephens, NSW (Australia) of which no aetiological agent was definitively identified were characterised (Chapter Two – published in Microbial Ecology) and provided fresh insight into the potential involvement of the microbiome. Third, the microbiomes of *C. gigas* bred for disease-resistance to the viral pathogen OsHV-1 μvar were characterised (Chapter Three – published in Frontiers in Microbiology). Chapter three identified host genetics driven by selection for OsHV-1 μvar-resistance as a factor influencing the *C. gigas* microbiome, and that *Vibrio* abundance was an indicative factor for disease-susceptibility in susceptible lines - a novel observation that strengthens support for the involvement of *Vibrio* bacteria in the OsHV-1 μvar disease process. Fourth, the microbiomes of oysters spanning six estuaries across 470 km were characterised to determine the broad- and individual-scale processes that shape the *C. gigas* microbiome (Chapter Four – under review). Chapter four identified bacterial taxa tied to the *C. gigas* microbiome, concluding that baseline microbiome profiles must be determined for individual culturing locations before microbiome shifts can be correctly interpreted. Fifth, a new molecular tool was developed to allow for enhanced characterisation of the *Vibrio*-specific community within a microbiome (Chapter Five – under review). Because of the significance of

Vibrio in diseases of oysters and other organisms, this new tool will be useful for identifying fine-scale species-level patterns in the *Vibrio* community and for identifying putative *Vibrio* pathogens during disease outbreaks. In this final discussion chapter (Chapter Six), three main points drawn from all the data chapters will be discussed. These are: i) factors affecting the microbiome composition in *C. gigas*, ii) *Vibrio* species and *C. gigas* disease dynamics, and iii) the future for using a microbiome approach in oyster health and disease.

6.1 Factors affecting the microbiome composition in *Crassostrea gigas*

While it is becoming increasingly apparent that the *C. gigas* microbiome is a contributing factor to disease processes (Wegner et al., 2013; Lemire et al., 2015; Lokmer and Wegner, 2015; Petton et al., 2015; De Lorgeril et al., 2018; Green et al., 2019; King et al., 2019a; King et al., 2019c), little is known about the factors that determine oyster microbiome composition thus hindering interpretations of the microbiome's potential contribution to health or disease susceptibility. Currently, studies have identified genetics, temperature, location, season, tissue-type and disease state as explanatory variables (Wegner et al., 2013; Wendling et al., 2014; Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b). The findings in this thesis support the literature by providing evidence that a field disease event (Chapter Two) and genetics (Chapter Three) alter the oyster microbiome. Further, while location has been identified as a factor influencing the microbiome, the findings of Chapter Four identified that the influence of location on the microbiome is, as expected, complex. As similarities between oyster microbiomes at geographically distant wave-dominated estuaries was observed, it can be hypothesised that estuaries with shared characteristics will influence the oyster microbiome in similar ways.

Despite the dynamic nature of the oyster microbiome (Wegner et al., 2013; Wendling et al., 2014; Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b) and substantial between-individual variation in the oyster adductor muscle microbiome (Chapters Two and Three) and gill microbiome (Wegner et al., 2013), core components of the oyster microbiome were successfully identified in Chapter Four for individual tissues and across all samples from geographically distant locations. Examinations of the core microbiome are important as they may indicate microbes that are important for host health (i.e. symbiotic) or in the functioning, or stabilisation of the microbial consortia (Shade and Handelsman, 2012). One particular core bacterial taxon identified in Chapter Four, an uncultured spirochete, was also identified in oyster microbiome research from Chapters Two, Three and in domestically and internationally published literature (Green and Barnes, 2010; Fernandez-Piquer et al., 2012; Lokmer et al., 2016a). Further, this uncultured spirochete was also identified in another oyster species (Green and Barnes, 2010). These data may indicate a symbiotic relationship of this taxa with oyster hosts and may be important in oyster health or as a 'keystone' bacterial species that helps stabilise the microbiome. To determine the importance of this core bacteria on oyster health, future studies should attempt to cultivate and characterise this uncultured spirochete. Whole genome sequencing could be used to identify the presence (or absence) of markers or pathways that indicate symbiosis. This may raise new research avenues regarding the suitability of this bacteria as a probiotic treatment for protecting or improving oyster health.

6.2 *Vibrio* species and *Crassostrea gigas* disease dynamics

Vibrio species are often implicated in oyster diseases (Garnier et al., 2007; Elston et al., 2008; Wendling et al., 2014; Lemire et al., 2015; Bruto et al., 2017; Go et al., 2017; Green et al., 2019) with shifts between commensalism to pathogenicity often facilitated by acquisition of a mobile genetic element(s) (Bruto et al., 2017) or with progressive replacement of commensal *Vibrios* with *Vibrio* pathogens preceding disease onset (Lemire et al., 2015). Further, small non-significant shifts in the *Vibrio* community in other marine organisms can alter the host's microbiome metabolism, affecting the health status of the host (Thurber et al., 2009). Consistent with the literature, an over-representation of *Vibrio* 16S rRNA reads was identified in disease-affected *C. gigas* microbiomes (Chapter Two) and in *C. gigas* with low resistance to OsHV-1 µvar (Chapter Three), the latter confirmed with a *Vibrio*-specific qPCR assay (Chapter Three).

During disease events, it is not unusual to identify an increase in *Vibrio* abundance in diseased oysters relative to healthy oysters (Lokmer and Wegner, 2015; Green et al., 2019; King et al., 2019a). The elevation in *Vibrio* abundance in diseased oysters is likely attributable to opportunistic *Vibrio* pathogens however, much of the microbiome work for oyster disease events has been performed in the laboratory (Wegner et al., 2013; Lemire et al., 2015; Lokmer and Wegner, 2015; Petton et al., 2015; Green et al., 2019) which is not representative of field conditions (Lokmer et al., 2016a). The elevation of *Vibrio* abundance could be attributable to the tank conditions providing a slight selective advantage to *Vibrio* species and allowing them to thrive. Therefore, measuring the level of *Vibrios* in oysters in the field is required to support these laboratory-based studies. Previous work performed on a summer mortality event in the Port Stephens estuary identified numerous putative *Vibrio* pathogens through standard culture-based

methodology (Go et al., 2017), specifically *V. harveyi* was over-represented (Go et al., 2017). Building upon this work, a microbiome approach was applied to this disease event (Chapter Two) which identified a specific elevation of reads assigned to *V. harveyi* and the *Vibrio* genus corresponding to the culture-based approach (Go et al., 2017) and further implicating *Vibrio* species in oyster diseases.

The results of Chapter Three identified that *Vibrio* abundance was significantly higher in healthy OsHV-1 disease-susceptible oysters. Breeding for disease resistance typically results in immunological changes (Bezemer et al., 2006) and this possibly allows the immune system to control growth of *Vibrio*. A larger *Vibrio* community within an oyster might increase the potential of a strain becoming pathogenic through acquisition of mobile DNA encoding virulence traits (Bruto et al., 2017) or allow for easier replacement of commensal vibrios with putative pathogens (Lemire et al., 2015). Infection by OsHV-1 μvar leads to immunosuppression of the host and allowing for opportunistic bacteria such as *Vibrio* to infect and cause disease (De Lorgeril et al., 2018). As the oyster microbiome can act as a source of pathogens (Wendling et al., 2014; Lokmer and Wegner, 2015), a larger *Vibrio* community may contain opportunistic pathogens that can infect the oyster during times of stress however, as current 16S rRNA sequencing technologies have poor phylogenetic power at the species level, it is unclear whether a larger *Vibrio* community also translates to an increase in *Vibrio* diversity.

Given the importance of *Vibrio* species in *C. gigas* disease dynamics, they are often studied using culture-dependent (Wendling et al., 2014; Lemire et al., 2015; Go et al., 2017) and culture-independent 16S rRNA techniques (Wegner et al., 2013; Lokmer and Wegner, 2015; Green et al., 2019; King et

al., 2019a; King et al., 2019c). Because of the poor species level resolution of 16S rRNA sequencing methods, it has been a challenge to identify specific *Vibrio* species potentially involved in the disease process. Therefore, a new high-throughput amplicon sequencing assay with an associated *Vibrio* database was developed in Chapter Five to provide superior taxonomic classification of *Vibrio* species and to provide more explanatory information when detangling the involvement of *Vibrio* species in disease. The developed *Vibrio*-specific *hsp60* assay was validated against a mock community constructed with ten different *Vibrio* species, all of which were identified with the *hsp60* assay and the utility of this sequencing assay was then demonstrated for a laboratory-induced summer mortality event (Green et al., 2019). The *hsp60* assay was able to identify shifts in the *Vibrio* community prior to the onset of disease and was able to unambiguously pinpoint *V. harveyi* as a putative pathogen for this disease event. This assay can be applied to other *C. gigas* diseases such as OsHV-1 μvar infection and summer mortality in which *Vibrio* presumptuously act as opportunistic pathogens contributing to the disease process (Go et al., 2017; De Lorgeril et al., 2018).

6.3 The future for using a microbiome approach

As previously mentioned, oyster microbiome studies are biased towards laboratory studies (Wegner et al., 2013; Lemire et al., 2015; Lokmer and Wegner, 2015; Petton et al., 2015; Green et al., 2019) which are unlikely to be representative of oysters in nature. Because of this, taxa identified in laboratory-based 16S rRNA datasets as potential contributing agents to disease could be over-estimated. For example, the findings in Chapter Three identified members of the *Tenacibaculum* genus as being associated with disease-resistance however, a previous study identified an elevation of reads

assigned to the *Tenacibaculum* genus following OsHV-1 µvar infection (De Lorgeril et al., 2018). The contrasting findings could be due to different methodological approaches (i.e. different 16S rRNA regions and laboratory versus field studies) or different regionally-separated *Tenacibaculum* species. But, could also be attributable to the effect of location on the oyster microbiome identified in Chapter Four, further compounding the difficulty in identifying putative pathogens. To overcome the influence of location on the microbiome, future studies will need to employ a high-resolution sampling regimen in the environment, coupled with bacterial culturing for whole genome sequencing (WGS) and both universal- and genera-specific sequencing assays (Chapter Five), to examine shifts in the oyster microbiome before, during and after disease. A longitudinal study which captures a 'healthy' baseline microbiome is necessary as the oyster microbiome is strongly influenced by location (Chapter Four) and examining shifts in the microbiome from other locations may lead to incorrect interpretations. Furthermore, developing a baseline microbiome profile may be necessary in places where oysters are cultivated to allow for disease state interpretations at later times.

While culturing and WGS can be useful when studying disease events by providing metabolic information, it should also be used to study the 'healthy' microbiome to identify members/metabolic functionality of the microbiome that may be influential for host health. Chapter Four identified a small core microbiome in healthy oysters across all locations and tissues, as well as a core microbiome for each individual tissue. The core microbes identified would be good targets for cultivation and WGS as they may have unique, possibly symbiotic, metabolic capabilities for the individual tissues they were identified in. In particular, an uncultured spirochete bacterium was identified in Chapters Two, Three and Four and in previously published

studies (Green and Barnes, 2010; Fernandez-Piquer et al., 2012; Lokmer et al., 2016a). Given the strong representation of this spirochete bacterium in multiple datasets, future studies should aim to culture these microorganisms for characterisation of their metabolic potential and employ techniques such as fluorescence in situ hybridization (FISH) and nanoscale secondary ion mass spectrometry (nanoSIMS) to determine the localisation of this spirochete and potential interactions this microbe has with the oyster host.

The future of oyster microbiome research is closely coupled to advances in sequencing technologies, such as metagenomics, allowing for the estimation of metabolic and virulence potential of a microbial community (Quince et al., 2017). Metagenomics could provide insights into metabolic shifts in the oyster microbiome prior to disease onset and whether virulence genes are elevated under periods of stress. However, a key challenge of using metagenomics in oyster research is the non-specific nature of the assay, effectively sequencing bulk DNA (i.e. host and microbial). Therefore, host DNA would need to be depleted from samples or the microbial DNA enriched prior to sequencing (Thoendel et al., 2016). Another new sequencing technique that could prove useful in the aquaculture field is the *Vibrio*-specific *hsp60* sequencing assay developed in Chapter Five. While it does not provide metabolic information about the *Vibrio* species, the assay can identify species level shifts within the *Vibrio* community, useful when attempting to unambiguously identify putative *Vibrio* pathogens. Identifying species level shifts is particular important for the *Vibrio* community, as small shifts in this community may be detrimental to the host (Thurber et al., 2009; Lemire et al., 2015).

Finally, and of most interest to the aquaculture industry, future studies should incorporate new statistical techniques such as network analysis for the

development of predictive frameworks for disease events. Performing a high-resolution sampling study with sufficient sampling before, during and after a disease even that captures a number of relevant factors would be useful for creating a predictive framework. Collected data could include a suite of abiotic factors (e.g. temperature, salinity, nutrients, pH) as well as oyster samples for subsequent DNA and RNA extractions. Oyster DNA could be used for high-throughput sequencing to examine shifts in bacterial taxa over time and to pinpoint specific microbial indicators of oyster health and disease. Oyster RNA could be used with new RNA counting technologies (e.g. nanostring) to identify markers of oyster health and to quantify their transcription. Abiotic data could be correlated to the oyster microbiome to explain shifts in the host response and microbial assemblage. Collected data can then be used with network analysis (e.g. extended local similarity analysis) to identify significant relationships between microbes, abiotic data and the host response, particularly if any time-delayed relationships exist (e.g. microbial patterns related to disease after rainfall events).

A simple example of how the collected data could fit within a predictive framework is provided below (Figure 6.1).

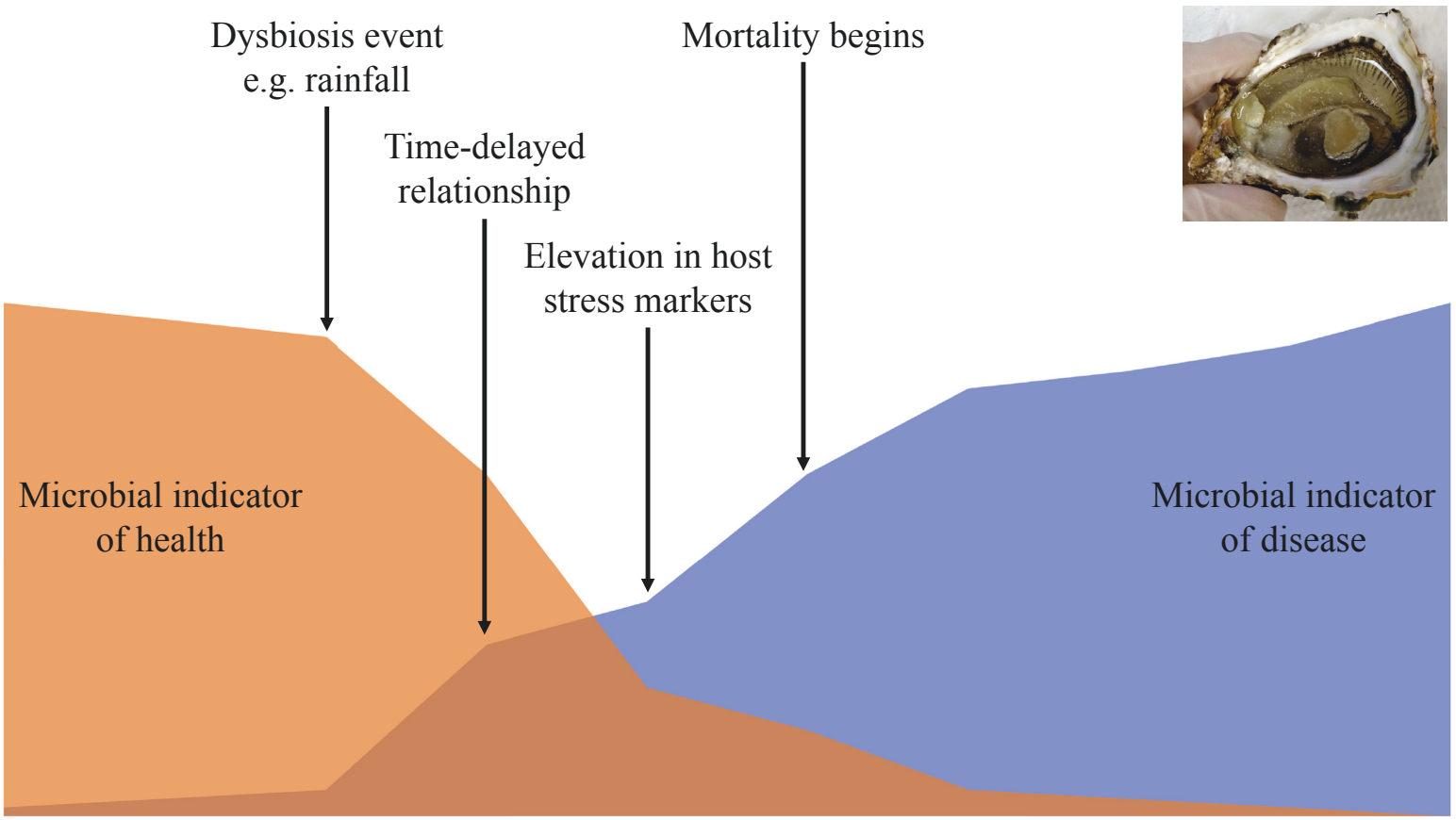


Figure 6.1: Theoretical predictive framework of oyster disease using high-throughput sequencing data (i.e. 16S rRNA), abiotic data, host immunological response and network analysis (i.e. extended local similarity analysis). Data would need to be collected during a high-resolution temporal study to capture a 'healthy' oyster baseline, followed by disease and eventual re-stabilisation.

In the aforementioned scenario, a microbial indicator of health and disease is identified and corresponds to respective decreases and increases in relative abundance. A dysbiosis event (e.g. rainfall or OsHV-1 infection) destabilises the oyster microbiome allowing for an opportunistic pathogen (i.e. microbial indicator of disease) to begin proliferating. A time-delayed relationship is observed between the abiotic driven dysbiosis event and opportunistic pathogen growth. An elevation in host stress and defense genes corresponds to opportunistic pathogen(s) proliferation before mortality is observed. The useable signals of disease risk identified with network analysis are: i) relative abundance decrease in the microbial indicator of health, ii) relative abundance increase in the microbial indicator of disease, iii) dysbiosis event (i.e. tracking rainfall) and iv) elevation in the host stress and defense response. Currently, apart from some abiotic factors, detecting these signals is not technologically feasible however, with sequencing technology becoming cheaper and more accessible, it may eventually be possible for oyster farmers to process samples following known trigger events (e.g. rainfall, temperature) to obtain data that helps assess risk and make decisions. By understanding how the oyster microbiome responds to disease and identifying the complex interaction of environmental parameters that correspond to disease – it is hoped that a robust framework for the prediction of oyster diseases be developed and used to minimise the impact of disease outbreaks.

7.0 References

Abares (2017). Australian fisheries and aquaculture statistics 2016. *Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES)* Department of Agriculture and Water Resources.

Abraham, C., and Cho, J.H. (2009). Inflammatory bowel disease. *New England Journal of Medicine* 361**,** 2066-2078.

Abs (2016). Census data. *Australian Bureau of Statistics*.

Adlard, R., and Ernst, I. (1995). Extended range of the oyster pathogen, *Marteilia sydneyi*. *Bulletin of the European Association of fish Pathologists* 15**,** 119-121.

Adlard, R.D., and Nolan, M.J. (2015). Elucidating the life cycle of *Marteilia sydneyi*, the aetiological agent of QX disease in the Sydney rock oyster (*Saccostrea glomerata*). *International Journal for Parasitology* 45**,** 419-426.

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., Woolsey, E.S., Bourne, D.G., Bongaerts, P., Hoegh-Guldberg, O., and Leggat, W. (2015). The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *ISME Journal* 9**,** 2261-2274.

Alderman, D. (1979). Epizootiology of *Marteilia refringens* in Europe. *Marine Fisheries Review* 41**,** 67-69.

Allam, B., Carden, W.E., Ward, J.E., Ralph, G., Winnicki, S., and Pales Espinosa, E. (2013). Early host-pathogen interactions in marine bivalves: evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. *Journal of Invertebrate Pathology* 113**,** 26-34.

Allam, B., and Pales Espinosa, E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? *Fish and Shellfish Immunology* 53**,** 4-12.

Amin, A.K.M.R., Feng, G., Al-Saari, N., Meirelles, P.M., Yamazaki, Y., Mino, S., Thompson, F.L., Sawabe, T., and Sawabe, T. (2016). The First Temporal and Spatial Assessment of *Vibrio* Diversity of the Surrounding Seawater of Coral Reefs in Ishigaki, Japan. *Frontiers in Microbiology* 7.

Anderson, T.J., Wesche, S., and Lester, R.J. (1994). Are outbreaks of *Marteilia sydneyi* in Sydney rock oysters, *Saccostrea commercialis*, triggered by a drop in environmental pH? *Australian Journal of Marine and Freshwater Research* 45**,** 1285-1287.

Andrews, J.D., and Hewatt, W.G. (1957). Oyster Mortality Studies in Virginia. II. The Fungus Disease Caused by *Dermocystidium marinum* in Oysters of Chesapeake Bay. *Ecological Monographs* 27**,** 2-25.

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

Arthur, R.F., Gurley, E.S., Salje, H., Bloomfield, L.S.P., and Jones, J.H. (2017). Contact structure, mobility, environmental impact and behaviour: The importance of social forces to infectious disease dynamics and disease ecology. *Philosophical Transactions of the Royal Society B: Biological Sciences* 372.

Arzul, I., Chollet, B., Boyer, S., Bonnet, D., Gaillard, J., Baldi, Y., Robert, M., Joly, J.Â.P., Garcia, C., and Bouchoucha, M. (2014). Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*. *Parasitology* 141**,** 227-240.

Arzul, I., Gagnaire, B., Bond, C., Chollet, B., Morga, B., Ferrand, S., Robert, M., and Renault, T. (2009). Effects of temperature and salinity on the

survival of *Bonamia ostreae*, a parasite infecting flat oysters *Ostrea edulis*. *Diseases of Aquatic Organisms* 85**,** 67-75.

Arzul, I., Langlade, A., Chollet, B., Robert, M., Ferrand, S., Omnes, E., Lerond, S., Couraleau, Y., Joly, J.P., François, C., and Garcia, C. (2011). Can the protozoan parasite *Bonamia ostreae* infect larvae of flat oysters *Ostrea edulis*? *Veterinary Parasitology* 179**,** 69-76.

Arzul, I., Renault, T., Lipart, C., and Davison, A.J. (2001). Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of General Virology* 82**,** 865-870.

Asi (2015). Australian Seafood Industries oyster breeding programs. *http://www.asioysters.com.au/breeding-program.html*.

Audemard, C., Barnaud, A., Collins, C.M., Le Roux, F., Sauriau, P.G., Coustau, C., Blachier, P., and Berthe, F.C.J. (2001). Claire ponds as an experimental model for *Marteilia refringens* life-cycle studies: New perspectives. *Journal of Experimental Marine Biology and Ecology* 257**,** 87-108.

Audemard, C., Le Roux, F., Barnaud, A., Collins, C., Sautour, B., Sauriau, P.G., De Montaudouin, X., Coustau, C., Combes, C., and Berthe, F. (2002). Needle in a haystack: Involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*. *Parasitology* 124**,** 315-323.

Austin, B., and Zhang, X.-H. (2006). *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Letters in Applied Microbiology* 43**,** 119-124.

Azéma, P., Travers, M.A., De Lorgeril, J., Tourbiez, D., and Dégremont, L. (2015). Can selection for resistance to OsHV-1 infection modify susceptibility to *Vibrio aestuarianus* infection in *Crassostrea gigas*? First insights from experimental challenges using primary and successive exposures. *Veterinary Research* 46.

Bachere, E.H., Dominique. Mialhe, Eric (1991). Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. *Diseases Of Aquatic Organisms* 11**,** 173-180.

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307**,** 1915-1920.

Balouet, G., Poder, M., and Cahour, A. (1983). Haemocytic parasitosis: Morphology and pathology of lesions in the French flat oyster, *Ostrea edulis* L. *Aquaculture* 34**,** 1-14.

Bäumler, A.J., and Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535**,** 85-93.

Becker, P., Gillan, D., Lanterbecq, D., Jangoux, M., Rasolofonirina, R., Rakotovao, J., and Eeckhaut, I. (2004). The skin ulceration disease in cultivated juveniles of *Holothuria scabra* (Holothuroidea, Echinodermata). *Aquaculture* 242**,** 13-30.

Ben-Haim, Y., and Rosenberg, E. (2002). A novel Vibrio sp. pathogen of the coral *Pocillopora damicornis*. *Marine Biology* 141**,** 47-55.

Berthe, F.C.J., Pernas, M., Zerabib, M., Haffner, P., Thébault, A., and Figueras, A.J. (1998). Experimental transmission of *Marteilia refringens* with special consideration of its life cycle. *Diseases of Aquatic Organisms* 34**,** 135-144.

Bezemer, B., Butt, D., Nell, J., Adlard, R., and Raftos, D. (2006). Breeding for QX disease resistance negatively selects one form of the defensive enzyme, phenoloxidase, in Sydney rock oysters. *Fish and Shellfish Immunology* 20**,** 627-636.

Boardman, C.L., Maloy, A.P., and Boettcher, K.J. (2008). Localization of the bacterial agent of juvenile oyster disease (*Roseovarius*

crassostreae) within affected eastern oysters (*Crassostrea virginica*). *Journal of Invertebrate Pathology* 97**,** 150-158.

Boettcher, K.J., Barber, B.J., and Singer, J.T. (1999). Use of antibacterial agents to elucidate the etiology of juvenile oyster disease (JOD) in *Crassostrea virginica* and numerical dominance of an α-proteobacterium in JOD-affected animals. *Applied and Environmental Microbiology* 65**,** 2534-2539.

Boettcher, K.J., Geaghan, K.K., Maloy, A.P., and Barber, B.J. (2005). *Roseovarius crassostreae* sp. nov., a member of the Roseobacter clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *International Journal of Systematic and Evolutionary Microbiology* 55**,** 1531-1537.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E., Da Silva, R., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., Mcdonald, D., Mciver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, I.I.M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R.,

Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., Van Der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., Von Hippel, M., Walters, W., Wan, Y., Wang, M., et al. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Preprints* 6**,** e27295v27291.

Bougrier, S., Geairon, P., Deslous-Paoli, J.M., Bacher, C., and Jonquières, G. (1995). Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). *Aquaculture* 134**,** 143-154.

Bower, S.M. (2006). Synopsis of infectious diseases and parasites of commercially exploited shellfish: Nocardiosis of oysters. *Fisheries and Oceans Canada*.

Bower, S.M. (2011). Synopsis of infectious diseases and parasites of commercially exploited shellfish: Marteiliosis (Aber Disease) of oysters. *Department of Fisheries and Oceans Canada*.

Bower, S.M., Hervio, D., and Meyer, G.R. (1997). Infectivity of *Mikrocytos mackini*, the causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas*, to various species of oysters. *Disease of Aquatic Organisms* 29**,** 111-116.

Boyd, J.N., and Burnett, L.E. (1999). Reactive oxygen intermediate production by oyster hemocytes exposed to hypoxia. *Journal of Experimental Biology* 202**,** 3135-3143.

Breitburg, D.L., Hondorp, D., Audemard, C., Carnegie, R.B., Burrell, R.B., Trice, M., and Clark, V. (2015). Landscape-Level Variation in Disease Susceptibility Related to Shallow-Water Hypoxia. *PLoS ONE* 10**,** e0116223.

Bricelj, V.M., Ford, S.E., Borerro, F.J., Perkins, F.O., Rivara, G., Hillman, R.E., Elston, R.A., and Chang, J. (1992). Unexplained mortalities of

hatchery-reared, juvenile oysters, *Crassostrea virginica* (Gmelin). *Journal of Shellfish Research* 11**,** 331-347.

Brinker, A., Pfeifer, G., Kerner, M.J., Naylor, D.J., Hartl, F.U., and Hayer-Hartl, M. (2001). Dual Function of Protein Confinement in Chaperonin-Assisted Protein Folding. *Cell* 107**,** 223-233.

Brodersen, K.E., Siboni, N., Nielsen, D.A., Pernice, M., Ralph, P.J., Seymour, J., and Kühl, M. (2018). Seagrass rhizosphere microenvironment alters plant-associated microbial community composition. *Environmental Microbiology* 20**,** 2854-2864.

Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., Polz, M.F., and Le Roux, F. (2017). *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME Journal* 11**,** 1043-1052.

Burge, C.A., Griffin, F.J., and Friedman, C.S. (2006). Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. *Diseases of Aquatic Organisms* 72**,** 31-43.

Burge, C.A., Judah, L.R., Conquest, L.L., Griffin, F.J., Cheney, D.P., Suhrbier, A., Vadopalas, B., Olin, P.G., Renault, T., and Friedman, C.S. (2007). Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: The influence of oyster stock, planting time, pathogens, and environmental stressors. *Journal of Shellfish Research* 26**,** 163-172.

Burreson, E.M., and Ragone Calvo, L.M. (1996). Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *Journal of Shellfish Research* 15**,** 17-34.

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

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods* 13**,** 581-583.

Camacho, A.F., Villalba, A., Beiras, R., and Labarta, U. (1997). Absorption efficiency and condition of cultured mussels (*Mytilus Edulis* Galloprovincialis Linnaeus) of Galicia (NW Spain) infected by parasites *Marteilia refringens* grizel et al. and *Mytilicola intestinalis* steuer. *Journal of Shellfish Research* 16**,** 77-82.

Camara, M.D., Yen, S., Kaspar, H.F., Kesarcodi-Watson, A., King, N., Jeffs, A.G., and Tremblay, L.A. (2017). Assessment of heat shock and laboratory virus challenges to selectively breed for ostreid herpesvirus 1 (OsHV-1) resistance in the Pacific oyster, *Crassostrea gigas*. *Aquaculture* 469**,** 50-58.

Canesi, L., Gallo, G., Gavioli, M., and Pruzzo, C. (2002). Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microscopy Research and Technique* 57**,** 469-476.

Cano-Gomez, A., Høj, L., Owens, L., and Andreakis, N. (2011). Multilocus sequence analysis provides basis for fast and reliable identification of *Vibrio harveyi*-related species and reveals previous misidentification of important marine pathogens. *Systematic and Applied Microbiology* 34**,** 561-565.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7**,** 335-336.

Carnegie, R.B., and Burreson, E.M. (2011). Declining impact of an introduced pathogen: -*Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. *Marine Ecology Progress Series* 432**,** 1-15.

Carnegie, R.B., Hill, K.M., Stokes, N.A., and Burreson, E.M. (2014). The haplosporidian *Bonamia exitiosa* is present in Australia, but the identity of the parasite described as *Bonamia* (formerly Mikrocytos) *roughleyi* is uncertain. *Journal of Invertebrate Pathology* 115**,** 33-40.

Cavanagh, D., Spurway, P., and Mcalister, T. (2004). *Batemans Bay and Clyde River Estuary Management Study* [Online]. Eurobodalla Shire Council. Available: http://www.esc.nsw.gov.au/living-in/living-sustainably/animals,-the-bush,-and-the-coast/estuaries-of-eurobodalla/batemans-bay,-clyde-river-estuary/batemans-bay-estuary-management-study-by-sections/ClydeEstuaryMgtStudy.pdf [Accessed 2019].

Chase, E., Young, S., and Harwood, V.J. (2015). Sediment and Vegetation as Reservoirs of *Vibrio vulnificus* in the Tampa Bay Estuary and Gulf of Mexico. *Applied and Environmental Microbiology* 81**,** 2489.

Chaudhary, D.K., and Kim, J. (2018). *Acidovorax monticola* sp. nov., isolated from soil. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 111**,** 1925-1934.

Chauhan, A., Wafula, D., Lewis, D.E., and Pathak, A. (2014). Metagenomic assessment of the Eastern oyster-associated microbiota. *Genome Announcements* 2.

Cheng, T.C., and Rodrick, G.E. (1975). Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comparative Biochemistry and Physiology* 52**,** 443-447.

Chimetto, L.A., Brocchi, M., Thompson, C.C., Martins, R.C., Ramos, H.R., and Thompson, F.L. (2008). Vibrios dominate as culturable nitrogen-

fixing bacteria of the Brazilian coral *Mussismilia hispida*. *Systematic and Applied Microbiology* 31**,** 312-319.

Choi, K.S., Wilson, E.A., Lewis, D.H., Powell, E.N., and Ray, S.M. (1989). The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. *Journal of Shellfish Research* 8**,** 125-131.

Chun, S.J., Cui, Y., Ko, S.R., Lee, H.G., Srivastava, A., Oh, H.M., and Ahn, C.Y. (2017). *Acidovorax lacteus* sp. nov., isolated from a culture of a bloom-forming cyanobacterium (*Microcystis sp.*). *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 110**,** 1199-1205.

Conway, J.R., Lex, A., and Gehlenborg, N. (2017). UpSetR: An R Package for the Visualization of Intersecting Sets and their Properties. *Bioinformatics*.

Cook, T., Folli, M., Klinck, J., Ford, S., and Miller, J. (1998). The relationship between increasing sea-surface temperature and the northward spread of *Perkinsus marinus* (Dermo) disease epizootics in oysters. *Estuarine, Coastal and Shelf Science* 46**,** 587-597.

Cotter, E., Malham, S.K., O'keeffe, S., Lynch, S.A., Latchford, J.W., King, J.W., Beaumont, A.R., and Culloty, S.C. (2010). Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of growth, biochemistry and gametogenesis. *Aquaculture* 303**,** 8-21.

Creeper, J.H., and Buller, N.B. (2006). An outbreak of *Streptococcus iniae* in barramundi (*Lates calcarifera*) in freshwater cage culture. *Australian Veterinary Journal* 84**,** 408-411.

Crump, B.C., Wojahn, J.M., Tomas, F., and Mueller, R.S. (2018). Metatranscriptomics and amplicon sequencing reveal mutualisms in seagrass microbiomes. *Frontiers in Microbiology* 9.

Cuadrado, V., Gomila, M., Merini, L., Giulietti, A.M., and Moore, E.R.B. (2010). *Cupriavidus pampae* sp. nov., a novel herbicide-degrading bacterium isolated from agricultural soil. *International Journal of Systematic and Evolutionary Microbiology* 60**,** 2606-2612.

Cúcio, C., Engelen, A.H., Costa, R., and Muyzer, G. (2016). Rhizosphere Microbiomes of European Seagrasses Are Selected by the Plant, But Are Not Species Specific. *Frontiers in Microbiology* 7**,** 440-440.

Da Costa, A.K.R., Pereira, L.C.C., Costa, S.F.S., Leite, N.R., De Flores-Montes, J.M., and Da Costa, R.M. (2016). Spatiotemporal variation in salinity during drought years in an Amazonian estuary (Taperaçu). *Journal of Coastal Research* 1**,** 48-52.

Daniels, N.A., and Shafaie, A. (2000). A review of pathogenic *Vibrio* infections for clinicians. *Infections in medicine* 17**,** 665-685.

Davison, A.J., Trus, B.L., Cheng, N., Steven, A.C., Watson, M.S., Cunningham, C., Le Deuff, R.M., and Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* 86**,** 41-53.

De Decker, S., and Saulnier, D. (2011). Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes. *Fish and Shellfish Immunology* 30**,** 691-699.

De Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., Haffner, P., Dégremont, L., Charrière, G.M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., Leroy, M.A., Pérignon, A., Régler, D., Morga, B., Alunno-Bruscia, M., Boudry, P., Le Roux, F., Destoumieux-Garzón, D., Gueguen, Y., and Mitta, G. (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nature Communications* 9**,** 4215.

Dégremont, L. (2011). Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. *Aquaculture* 317**,** 94-98.

Dégremont, L. (2013). Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. *Aquaculture* 416-417**,** 129-134.

Dégremont, L., Lamy, J.B., Pépin, J.F., Travers, M.A., and Renault, T. (2015a). New insight for the genetic evaluation of resistance to ostreid herpesvirus infection, a worldwide disease, in *Crassostrea gigas*. *PLoS ONE* 10.

Dégremont, L., Ledu, C., Maurouard, E., Nourry, M., and Benabdelmouna, A. (2016a). Effect of ploidy on the mortality of *Crassostrea gigas* spat caused by OsHV-1 in France using unselected and selected OsHV-1 resistant oysters. *Aquaculture Research* 47**,** 777-786.

Dégremont, L., Morga, B., Trancart, S., and Pépin, J.F. (2016b). Resistance to OsHV-1 infection in *crassostrea gigas* larvae. *Frontiers in Marine Science* 3.

Dégremont, L., Nourry, M., and Maurouard, E. (2015b). Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: Response to selection after four generations. *Aquaculture* 446**,** 111-121.

Dégremont, L., Tanguy, G., Delphine, T., and Jean-François, P. (2013). Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles? *Aquaculture* 408–409**,** 51-57.

Donovan, T.J., and Van Netten, P. (1995). Culture media for the isolation and enumeration of pathogenic *Vibrio* species in foods and environmental samples. *International Journal of Food Microbiology* 26**,** 77-91.

Dorsch, M., Lane, D., and Stackebrandt, E. (1992). Towards a phylogeny of the genus *Vibrio* based on 16S rRNA sequences. *International Journal of Systematic Bacteriology* 42**,** 58-63.

Dove, M.C., Nell, J.A., Mcorrie, S., and O'connor, W.A. (2013a). 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. *Journal of Shellfish Research* 32**,** 681-687.

Dove, M.C., Nell, J.A., and O'connor, W.A. (2013b). 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*). *Aquaculture Research* 44**,** 1791-1800.

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

Dove, M.C., and Sammut, J. (2007b). Impacts of estuarine acidification on survival and growth of Sydney rock oysters Saccostrea glomerata (Gould 1850). *Journal of Shellfish Research* 26**,** 519-527.

Dpi (2019). Aquaculture Production Report 2017-2018. *NSW Department of Primary Industries*.

Drexler, J.Z., and Ewel, K.C. (2001). Effect of the 1997-1998 ENSO-related drought on hydrology and salinity in a Micronesian wetland complex. *Estuaries* 24**,** 347-356.

Dubert, J., Barja, J.L., and Romalde, J.L. (2017). New Insights into Pathogenic Vibrios Affecting Bivalves in Hatcheries: Present and Future Prospects. *Frontiers in Microbiology* 8**,** 762-762.

Duperthuy, M., Schmitt, P., Garzón, E., Caro, A., Rosa, R.D., Le Roux, F., Lautrédou-Audouy, N., Got, P., Romestand, B., De Lorgeril, J.,

Kieffer-Jaquinod, S., Bachère, E., and Destoumieux-Garzón, D. (2011). Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proceedings of the National Academy of Sciences of the United States of America* 108**,** 2993-2998.

Earley, Z.M., Akhtar, S., Green, S.J., Naqib, A., Khan, O., Cannon, A.R., Hammer, A.M., Morris, N.L., Li, X., Eberhardt, J.M., Gamelli, R.L., Kennedy, R.H., and Choudhry, M.A. (2015). Burn injury alters the intestinal microbiome and increases gut permeability and bacterial translocation. *PLoS ONE* 10.

Earthtech (2008). Tilligerry Creek Management Plan. *Port Stephens Council*.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26**,** 2460-2461.

Egidius, E., Wiik, R., Andersen, K., Hoff, K.A., and Hjeltnes, B. (1986). *Vibrio salmonicida* sp. nov., a New Fish Pathogen. *International Journal of Systematic and Evolutionary Microbiology* 36**,** 518-520.

Elston, R., Friedman, C., Gustafson, L., Meyer, G., and Rogers, R. (2015). Denman Island disease in Washington State, USA: Distribution and prevalence in Pacific and Olympia oysters. *Diseases of Aquatic Organisms* 114**,** 147-154.

Elston, R.A., Farley, C. A. And Kent, M. L. (1986). Occurrence and significance of bonamiasis in European flat oysters *Ostrea edulis* in North America. *Diseases of Aquatic Organisms* 2**,** 49-54.

Elston, R.A., Hasegawa, H., Humphrey, K.L., Polyak, I.K., and Hase, C.C. (2008). Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: Severity, environmental drivers, geographic extent and management. *Diseases of Aquatic Organisms* 82**,** 119-134.

Embar-Gopinath, S., Butler, R., and Nowak, B. (2005). Influence of salmonid gill bacteria on development and severity of amoebic gill disease. *Diseases of Aquatic Organisms* 67**,** 55-60.

Embar-Gopinath, S., Crosbie, P., and Nowak, B.F. (2006). Concentration effects of *Winogradskyella* sp. on the incidence and severity of amoebic gill disease. *Diseases of Aquatic Organisms* 73**,** 43-47.

Encomio, V.G., Stickler, S.M., Allen Jr, S.K., and Chu, F.L. (2005). Performance of "natural dermo-resistant" oyster stocks - Survival, disease, growth, condition and energy reserves. *Journal of Shellfish Research* 24**,** 143-155.

Engelsma, M.Y., Roozenburg, I., and Joly, J.P. (2008). First isolation of *Nocardia crassostreae* from Pacific oyster *Crassostrea gigas* in Europe. *Diseases of Aquatic Organisms* 80**,** 229-234.

Estrada-De Los Santos, P., Solano-Rodríguez, R., Matsumura-Paz, L.T., Vásquez-Murrieta, M.S., and Martínez-Aguilar, L. (2014). *Cupriavidus plantarum* sp. nov., a plant-associated species. *Archives of Microbiology* 196**,** 811-817.

Ewart, J.W., and Ford, S.E. (1993). History and Impact of MSX and Dermo Diseases on Oyster Stocks In the Northeast Region. *Northeastern Regional Aquaculture Center, University of Massachusett*.

Fao (2016a). Cultured Aquatic Species Information Programme. *Crassostrea gigas*. Cultured Aquatic Species Information Programme. *FAO Fisheries and Aquaculture Department [online]. Text by Helm, M.M.*

Fao (2016b). Cultured Aquatic Species Information Programme. *Crassostrea virginica*. Cultured Aquatic Species Information Programme. *FAO Fisheries and Aquaculture Department [online]. Text by Kennedy, V. S.*

Fao (2016c). Cultured Aquatic Species Information Programme. *Ostrea edulis*. Cultured Aquatic Species Information Programme. *FAO Fisheries and Aquaculture Department [online]. Text by Goulletquer, P.*

Fao (2016d). Cultured Aquatic Species Information Programme. *Saccostrea commercialis*. Cultured Aquatic Species Information Programme. *FAO Fisheries and Aquaculture Department [online]. Text by John, A.N.*

Fao (2016e). Hatchery culture of bivalves: A practical manual. *FAO Fisheries and Aquaculture Department [online]. Text by Helm, M.M. and Bourne, N.*

Farley, C.A., Wolf, P.H., and Elston, 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.). *Fishery Bulletin* 86**,** 581-593.

Farr, G.W., Furtak, K., Rowland, M.B., Ranson, N.A., Saibil, H.R., Kirchhausen, T., and Horwich, A.L. (2000). Multivalent Binding of Nonnative Substrate Proteins by the Chaperonin GroEL. *Cell* 100**,** 561-573.

Fernandez-Piquer, J., Bowman, J.P., Ross, T., and Tamplin, M.L. (2012). Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure. *Journal of Applied Microbiology* 112**,** 1134-1143.

Florez, L.V., Biedermann, P.H., Engl, T., and Kaltenpoth, M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Nature Product Reports* 32**,** 904-936.

Ford, S., Powell, E., Klinck, J., and Hofmann, E. (1999). Modeling the MSX parasite in eastern oyster (*Crassostrea virginica*) populations. I.

Model development, implementation, and verification. *Journal of Shellfish Research* 18**,** 475-500.

Ford, S.E. (1996). Range extension by the oyster parasite P*erkinsus marinus* into the northeastern United States: response to climate change? *Journal of Shellfish Research* 15**,** 45-56.

Ford, S.E., and Haskin, H.H. (1982). History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen in Delaware Bay, 1957-1980. *Journal of Invertebrate Pathology* 40**,** 118-141.

Franco, A., Busse, H.J., Schubert, P., Wilke, T., Kämpfer, P., and Glaeser, S.P. (2018). *Winogradskyella pocilloporae* sp. Nov. Isolated from healthy tissue of the coral *Pocillopora damicornis*. *International Journal of Systematic and Evolutionary Microbiology* 68**,** 1689-1696.

Frank, D.N., St. Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N., and Pace, N.R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America* 104**,** 13780-13785.

Frans, I., Michiels, C.W., Bossier, P., Willems, K., Lievens, B., and Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of fish diseases* 34**,** 643-661.

Friedman, C.S., Beattie, J.H., Elston, R.A., and Hedrick, R.P. (1991). Investigation of the relationship between the presence of a Gram-positive bacterial infection and summer mortality of the Pacific oyster, *Crassostrea gigas* Thunberg. *Aquaculture* 94**,** 1-15.

Friedman, C.S., Estes, R.M., Stokes, N.A., Burge, C.A., Hargove, J.S., Barber, B.J., Elston, R.A., Burreson, E.M., and Reece, K.S. (2005). Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. *Diseases of Aquatic Organisms* 63**,** 33-41.

Friedman, C.S., and Hedrick, R.P. (1991). Pacific oyster nocardiosis: Isolation of the bacterium and induction of laboratory infections. *Journal of Invertebrate Pathology* 57**,** 109-120.

Friedman, C.S., and Perkins, F.O. (1994). Range extension of *Bonamia ostreae* to Maine, U.S.A. *Journal of Invertebrate Pathology* 64**,** 179-181.

Fujiya, M. (1970). Oyster farming in Japan. *Helgoländer Wissenschaftliche Meeresuntersuchungen* 20**,** 464-479.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., Taylor, T.D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M., and Ohno, H. (2011). *Bifidobacteria* can protect from enteropathogenic infection through production of acetate. *Nature* 469**,** 543-547.

Gagnaire, B., Duchemin, M., Auffret, M., Thomas-Guyon, H., and 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. *Aquatic Living Resources* 21**,** 39-43.

Garcia, C., Thebault, A., Degremont, L., Arzul, I., Miossec, L., Robert, M., Chollet, B., Francois, C., Joly, J.P., Ferrand, S., Kerdudou, N., and Renault, T. (2011). Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. *Vet Res* 42**,** 73.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., and Nicolas, J.L. (2007). Evidence for the involvement of pathogenic bacteria in summer mortalities of the pacific oyster *Crassostrea gigas*. *Microbial Ecology* 53**,** 187-196.

Gay, M., Renault, T., Pons, A.M., and Le Roux, F. (2004). Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*:

Taxonomy and host alterations. *Diseases of Aquatic Organisms* 62**,** 65-74.

Geng, Y., Liu, D., Han, S., Zhou, Y., Wang, K.Y., Huang, X.L., Chen, D.F., Peng, X., and Lai, W.M. (2014). Outbreaks of vibriosis associated with *Vibrio mimicus* in freshwater catfish in China. *Aquaculture* 433**,** 82-84.

Geyer, W.R. (1997). Influence of wind on dynamics and flushing of shallow estuaries. *Estuarine, Coastal and Shelf Science* 44**,** 713-722.

Gilad, O., Yun, S., Adkison, M.A., Way, K., Willits, N.H., Bercovier, H., and Hedrick, R.P. (2003). Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology* 84**,** 2661-2668.

Go, J., Deutscher, A., Spiers, Z., Dahle, K., Kirkland, P., and Jenkins, C. (2017). An investigation into mass mortalities of unknown aetiology in Pacific oysters, *Crassostrea gigas*, in Port Stephens, New South Wales, Australia. *Diseases of Aquatic Organisms* 125**,** 227-242.

Goarant, C., Herlin, J., Brizard, R., Marteau, A.-L., Martin, C., and Martin, B. (2000). Toxic factors of *Vibrio* strains pathogenic to shrimp. *Diseases of aquatic organisms* 40**,** 101-107.

Golosova, O., Henderson, R., Vaskin, Y., Gabrielian, A., Grekhov, G., Nagarajan, V., Oler, A.J., Quiñones, M., Hurt, D., and Fursov, M. (2014). Unipro UGENE NGS pipelines and components for variant calling, RNA-seq and ChIP-seq data analyses. *PeerJ* 2**,** e644.

Gomez-Gil, B., Soto-Rodriguez, S., Garcia-Gasca, A., Roque, A., Vazquez-Juarez, R., Thompson, F.L., and Swings, J. (2004). Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. *Microbiology* 150**,** 1769-1777.

Green, T.J., and Barnes, A.C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology* 109**,** 613-622.

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

Green, T.J., Siboni, N., King, W.L., Labbate, M., Seymour, J.R., and Raftos, D. (2019). Simulated Marine Heat Wave Alters Abundance and Structure of *Vibrio* Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event. *Microbial Ecology* 77**,** 736-747.

Grimes, D.J., Johnson, C.N., Dillon, K.S., Flowers, A.R., Noriea, N.F., and Berutti, T. (2009). What Genomic Sequence Information Has Revealed About *Vibrio* Ecology in the Ocean—A Review. *Microbial Ecology* 58**,** 447-460.

Grisez, L., Reyniers, J., Verdonck, L., Swings, J., and Ollevier, F. (1997). Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. *Aquaculture* 155**,** 387-399.

Hada, H.S., West, P.A., Lee, J.V., Stemmler, J., and Colwell, R.R. (1984). *Vibrio tubiashii* sp. nov., a Pathogen of Bivalve Mollusks. *International Journal of Systematic and Evolutionary Microbiology* 34**,** 1-4.

Hahn, M.W., Lang, E., Brandt, U., Lunsdorf, H., Wu, Q.L., and Stackebrandt, E. (2010). *Polynucleobacter cosmopolitanus* sp. nov., free-living planktonic bacteria inhabiting freshwater lakes and rivers. *International Journal of Systematic and Evolutionary Microbiology* 60**,** 166-173.

Hammer, Ø., Harper, D.a.T., and Ryan, P.D. (2001). Past: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4.

Handelsman, J. (2004). Metagenomics: Application of Genomics to Uncultured Microorganisms. *Microbiology and Molecular Biology Reviews* 68**,** 669-685.

Harlin, M.M. (1973). Transfer of products between epiphytic marine algae and host plants. *Journal of Phycology* 9**,** 243-248.

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., and Samuel, M.D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* 296**,** 2158-2162.

Haskin, H.H., and Ford, S.E. (1982). *Haplosporidium nelsoni* (MSX) on delaware bay seed oyster beds: A host-parasite relationship along a salinity gradient. *Journal of Invertebrate Pathology* 40**,** 388-405.

Haskin, H.H., Stauber, L.A., and Mackin, J.A. (1966). *Minchinia nelsoni* n. sp. (*haplosporida*, *haplosporidiidae*): Causative agent of the delaware bay oyster epizoötic. *Science* 153**,** 1414-1416.

Heckmann, K., and Schmidt, H.J. (1987). *Polynucleobacter necessarius* gen. nov., sp. nov., an obligately endosymbiotic bacterium living in the cytoplasm of *Euplotes aediculatus*. *International Journal of Systematic and Evolutionary Microbiology* 37**,** 456-457.

Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America* 108**,** 3047-3052.

Hemmingsen, S.M., Woolford, C., Van Der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W., and Ellis, R.J. (1988).

Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 333**,** 330.

Herlemann, D.P., Labrenz, M., Jurgens, K., Bertilsson, S., Waniek, J.J., and Andersson, A.F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME journal* 5**,** 1571-1579.

Hervio, D., Bower, S.M., and Meyer, G.R. (1996). Detection, isolation, and experimental transmission of *Mikrocytos mackini*, a microcell parasite of pacific oysters *Crassostrea gigas* (Thunberg). *Journal of Invertebrate Pathology* 67**,** 72-79.

Hill, J.E., Penny, S.L., Crowell, K.G., Goh, S.H., and Hemmingsen, S.M. (2004). cpnDB: a chaperonin sequence database. *Genome research* 14**,** 1669-1675.

Hine, P., Wesney, B., and Hay, B. (1992). Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. *Diseases of Aquatic Organisms* 12**,** 135-142.

Hood, M.A., and Winter, P.A. (1997). Attachment of *Vibrio cholerae* under various environmental conditions and to selected substrates. *FEMS Microbiology Ecology* 22**,** 215-223.

Hosokawa, T., Kikuchi, Y., Shimada, M., and Fukatsu, T. (2008). Symbiont acquisition alters behaviour of stinkbug nymphs. *Biology Letters* 4**,** 45-48.

Hunt, D.E., Gevers, D., Vahora, N.M., and Polz, M.F. (2008). Conservation of the chitin utilization pathway in the *Vibrionaceae*. *Applied and Environmental Microbiology* 74**,** 44-51.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rahman, R., and Colwell, R.R. (1983). Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Applied and Environmental Microbiology* 45**,** 275-283.

Itoh, N., and Takahashi, K.G. (2008). Distribution of multiple peptidoglycan recognition proteins in the tissues of Pacific oyster, *Crassostrea gigas*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 150**,** 409-417.

Jeffries, V.E. (1982). Three Vibrio strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. *Aquaculture* 29**,** 201-226.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'connor, W., Kirkland, P.D., and Frances, J. (2013). Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in *Crassostrea gigas* (Pacific oysters) in Australia. *Dis Aquat Organ* 105**,** 109-126.

Jensen, S.I., Kuhl, M., and Prieme, A. (2007). Different bacterial communities associated with the roots and bulk sediment of the seagrass *Zostera marina*. *FEMS Microbiol Ecol* 62**,** 108-117.

Jesser, K.J., and Noble, R.T. (2018). *Vibrio* ecology in the Neuse River Estuary, North Carolina, characterized by Next-Generation Amplicon Sequencing of the Gene Encoding Heat Shock Protein 60 (*hsp60*). *Applied and Environmental Microbiology*.

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

Kahlke, T. (2018). Ampli-Tools (Version 1.0). *Zenodo*.

Kahlke, T., and Ralph, P.J. (2019). BASTA – Taxonomic classification of sequences and sequence bins using last common ancestor estimations. *Methods in Ecology and Evolution* 10**,** 100-103.

Kapareiko, D., Lim, H.J., Schott, E.J., Hanif, A., and Wikfors, G.H. (2011). Isolation and Evaluation of New Probiotic Bacteria for use in Shellfish Hatcheries: II. Effects of a *Vibrio* sp. Probiotic Candidate Upon Survival of Oyster Larvae (*Crassostrea virginica*) in Pilot-Scale Trials. *Journal of Shellfish Research* 30**,** 617-625.

Katoh, K., Rozewicki, J., and Yamada, K.D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* bbx108.

Keeling, S.E., Brosnahan, C.L., Williams, R., Gias, E., Hannah, M., Bueno, R., Mcdonald, W.L., and Johnston, C. (2014). New Zealand juvenile oyster mortality associated with ostreid herpesvirus 1-an opportunistic longitudinal study. *Diseases of Aquatic Organisms* 109**,** 231-239.

Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., and Conway, S. (2004). Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol* 5**,** 104-112.

Keppel, A.G., Breitburg, D.L., Wikfors, G.H., Burrell, R.B., and Clark, V.M. (2015). Effects of co-varying diel-cycling hypoxia and pH on disease susceptibility in the eastern oyster *Crassostrea virginica*. *Marine Ecology Progress Series* 538**,** 169-183.

Kimes, N.E., Johnson, W.R., Torralba, M., Nelson, K.E., Weil, E., and Morris, P.J. (2013). The *Montastraea faveolata* microbiome: Ecological and temporal influences on a Caribbean reef-building coral in decline. *Environmental Microbiology* 15**,** 2082-2094.

King, G.M., Judd, C., Kuske, C.R., and Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One* 7**,** e51475.

King, W.L., Jenkins, C., Go, J., Siboni, N., Seymour, J.R., and Labbate, M. (2019a). Characterisation of the Pacific oyster microbiome during a summer mortality event. *Microbial Ecology* 77**,** 502-512.

King, W.L., Jenkins, C., Seymour, J.R., and Labbate, M. (2019b). Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment. *Marine Environmental Research* 143**,** 124-140.

King, W.L., Siboni, N., Williams, N.L.R., Kahlke, T., Nguyen, K.V., Jenkins, C., Dove, M., O'connor, W., Seymour, J.R., and Labbate, M. (2019c). Variability in the composition of Pacific Oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 µvar disease. *Frontiers in Microbiology* 10**,** 473.

Kleeman, S.N., Adlard, R.D., and 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. *International Journal for Parasitology* 32**,** 767-784.

Koch, H., and Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences* 108**,** 19288-19292.

Koch, R. (1884). "Die Ätiologie der Tuberkulose". Mittheilungen aus dem Kaiserlichen Gesundheitsamte).

Kreader, C.A. (1995). Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Applied and Environmental Microbiology* 61**,** 1171-1179.

Kube, P., Dove, M., Cunningham, M., Kirkland, P., Gu, X., Hick, P., O'connor, W., and Elliott, N. (2018). *Genetic Selection for Resistance to Pacific Oyster Mortality Syndrome* [Online]. Available: http://www.asioysters.com.au/uploads/2/5/9/7/25976125/kube_et_al__2018__genetic_selection_for_resistance_to_poms_-_sfcrc_project_final_report.pdf [Accessed 15 December 2018].

Kushmaro, A., Banin, E., Loya, Y., Stackebrandt, E., and Rosenberg, E. (2001). *Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*. *International journal of systematic and evolutionary microbiology* 51**,** 1383-1388.

Kwok, A.Y.C., Wilson, J.T., Coulthart, M., Ng, L.K., Mutharia, L., and Chow, A.W. (2002). Phylogenetic study and identification of human

pathogenic *Vibrio* species based on partial *hsp60* gene sequences. *Canadian Journal of Microbiology* 48**,** 903-910.

Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., and Nicolas, J.-L. (2006a). Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. *Microbes and Infection* 8**,** 2715-2724.

Labreuche, Y., Soudant, P., Goncalves, M., Lambert, C., and Nicolas, J.L. (2006b). Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and cellular immune responses of the oyster *Crassostrea gigas*. *Dev Comp Immunol* 30**,** 367-379.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M., and Poulet, S.A. (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**,** 139-145.

Lafferty, K.D., Harvell, C.D., Conrad, J.M., Friedman, C.S., Kent, M.L., Kuris, A.M., Powell, E.N., Rondeau, D., and Saksida, S.M. (2015). Infectious diseases affect marine fisheries and aquaculture economics. *Annual Review of Marine Science* 7**,** 471-496.

Lane, D.J. (1991). 16S/23S rRNA sequencing. *In: Nucleic acid techniques in bacterial systematics, Stackebrandt, E., and Goodfellow, M., eds., John Wiley and Sons, New York, NY***,** 115-175.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences* 82**,** 6955-6959.

Lang, R.P., Langdon, C., Taris, N., and Camara, M. (2010). *Use of laboratory assays to predict subsequent growth and survival of Pacific oyster (Crassostrea gigas) families planted in coastal waters.*

Le Roux, F., Wegner, K.M., and Polz, M.F. (2016). Oysters and Vibrios as a Model for Disease Dynamics in Wild Animals. *Trends in Microbiology* 24**,** 568-580.

Lee, J.H., Kang, J.W., Shin, S.B., and Seong, C.N. (2017). *Winogradskyella flava* sp. nov., isolated from the brown alga, *Sargassum fulvellum*. *International Journal of Systematic and Evolutionary Microbiology* 67**,** 3540-3546.

Lee, K.-H., and Ruby, E.G. (1994). Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Applied and environmental microbiology* 60**,** 1565-1571.

Lee, Y.S., Baik, K.S., Park, S.Y., Kim, E.M., Lee, D.H., Kahng, H.Y., Jeon, C.O., and Jung, J.S. (2009). *Tenacibaculum crassostreae* sp. nov., isolated from the Pacific oyster, *Crassostrea gigas*. *International Journal of Systematic and Evolutionary Microbiology* 59**,** 1609-1614.

Lehnen, N., Marchant, H.K., Schwedt, A., Milucka, J., Lott, C., Weber, M., Dekaezemacker, J., Seah, B.K., Hach, P.F., Mohr, W., and Kuypers, M.M. (2016). High rates of microbial dinitrogen fixation and sulfate reduction associated with the Mediterranean seagrass *Posidonia oceanica*. *Syst Appl Microbiol* 39**,** 476-483.

Lemire, A., Goudenege, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., and Le Roux, F. (2015). Populations, not clones, are the unit of *vibrio* pathogenesis in naturally infected oysters. *ISME Journal* 9**,** 1523-1531.

Lester, R.J.G. (1986). Field and laboratory observations on the oyster parasite *Marteilia sydneyi*. *University of Queensland Press* Parasite lives: Papers on parasites, their hosts and their associations**,** 33-40.

Leung, T.L.F., and Bates, A.E. (2013). More rapid and severe disease outbreaks for aquaculture at the tropics: implications for food security. *Journal of Applied Ecology* 50**,** 215-222.

Lewis, E.J., Farley, C.A., Small, E.B., and Baya, A.M. (1996). A synopsis of juvenile oyster disease (JOD) experimental studies in *Crassostrea virginica*. *Aquatic Living Resources* 9**,** 169-178.

Lin, B., Wang, Z., Malanoski, A.P., O'grady, E.A., Wimpee, C.F., Vuddhakul, V., Alves, N., Jr., Thompson, F.L., Gomez-Gil, B., and Vora, G.J. (2010). Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environmental microbiology reports* 2**,** 81-89.

Lipovsky, V.P., Chew, K.K. (1972). Mortality of Pacific oysters (*Crassostrea gigas*): the influence of temperature and enriched seawater on oyster survival. *Proc. Nat. Shellfish Ass***,** 72-82.

Lisansky, S.G. (1988). Fungi in biological control systems. *Edited by M. N. Burge, Manchester University Press*.

Liu, F., Liu, G., and Li, F. (2016). Characterization of two pathogenic *Photobacterium* strains isolated from *Exopalaemon carinicauda* causing mortality of shrimp. *Aquaculture* 464**,** 129-135.

Löffler, F. (1884). *Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe.*

Lokmer, A., Goedknegt, M.A., Thieltges, D.W., Fiorentino, D., Kuenzel, S., Baines, J.F., and Wegner, K.M. (2016a). Spatial and temporal dynamics of Pacific oyster hemolymph microbiota across multiple scales. *Frontiers in Microbiology* 7**,** 1367.

Lokmer, A., Kuenzel, S., Baines, J.F., and Wegner, K.M. (2016b). The role of tissue-specific microbiota in initial establishment success of Pacific oysters. *Environmental Microbiology* 18**,** 970-987.

Lokmer, A., and Wegner, K.M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME Journal* 9**,** 670-682.

López-Sanmartín, M., Batista, F.M., Del Carmen Marín, M., Garrido, I., Quintero, D., Grade, A., Ruano, F., De La Herrán, R., and Navas, J.I. (2015). Detection of *Marteilia refringens* infecting the European flat oyster *Ostrea edulis* and the dwarf oyster *Ostrea stentina* in southern Portugal and Spain. *Journal of Invertebrate Pathology* 130**,** 52-55.

Lopez-Sanmartin, M., Lopez-Fernandez, J.R., Cunha, M.E., De La Herran, R., and Navas, J.I. (2016). Ostreid herpesvirus in wild oysters from the Huelva coast (SW Spain). *Dis Aquat Organ* 120**,** 231-240.

Luna-González, A., Maeda-Martínez, A.N., Sainz, J.C., and Ascencio-Valle, F. (2002). Comparative susceptibility of veliger larvae of four bivalve mollusks to a *Vibrio alginolyticus* strain. *Diseases of Aquatic Organisms* 49**,** 221-226.

Lynch, S.A., Flannery, G., Hugh-Jones, T., Hugh-Jones, D., and Culloty, S.C. (2014). Thirty-year history of Irish (Rossmore) *Ostrea edulis* selectively bred for disease resistance to *Bonamia ostreae*. *Diseases of Aquatic Organisms* 110**,** 113-121.

Mackin, J.G. (1959). Mortalities in oysters. *Proceedings of the National Shellfisheries Association* 50**,** 21-40.

Mackin, J.G., Owen, H.M., and Collier, A. (1950). Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science* 111**,** 328-329.

Magoc, T., and Salzberg, S.L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27**,** 2957-2963.

Magoč, T., and Salzberg, S.L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27**,** 2957-2963.

Malham, S.K., Cotter, E., O'keeffe, S., Lynch, S., Culloty, S.C., King, J.W., Latchford, J.W., and Beaumont, A.R. (2009). Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of temperature and nutrients on health and survival. *Aquaculture* 287**,** 128-138.

Maloy, A.P., Ford, S.E., Karney, R.C., and Boettcher, K.J. (2007). *Roseovarius crassostreae*, the etiological agent of Juvenile Oyster Disease (now to be known as Roseovarius Oyster Disease) in *Crassostrea virginica*. *Aquaculture* 269**,** 71-83.

Marcelino, V.R., Van Oppen, M.J.H., and Verbruggen, H. (2018). Highly structured prokaryote communities exist within the skeleton of coral colonies. *ISME Journal* 12**,** 300-303.

Marshall, R., Mckinley, S., and Pearce, C.M. (2010). Effects of nutrition on larval growth and survival in bivalves. *Reviews in Aquaculture* 2**,** 33-55.

Martenot, C., Oden, E., Travaille, E., Malas, J.P., and Houssin, M. (2011). Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. *Virus Res* 160**,** 25-31.

Martinez-Urtaza, J., Bowers, J.C., Trinanes, J., and Depaola, A. (2010). Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. *Food Research International* 43**,** 1780-1790.

Mccallum, H., Harvell, D., and Dobson, A. (2003). Rates of spread of marine pathogens. *Ecology Letters* 6**,** 1062-1067.

Mcdonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., Desantis, T.Z., Probst, A., Andersen, G.L., Knight, R., and Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME Journal* 6**,** 610-618.

Miyashiro, T., and Ruby, E.G. (2012). Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Molecular microbiology* 84**,** 795-806.

Montes, J., Anadón, R., and Azevedo, C. (1994). A Possible Life Cycle for *Bonamia ostreae* on the Basis of Electron Microscopy Studies. *Journal of Invertebrate Pathology* 63**,** 1-6.

Moreau, P., Moreau, K., Segarra, A., Tourbiez, D., Travers, M.A., Rubinsztein, D.C., and Renault, T. (2015). Autophagy plays an important role in protecting Pacific oysters from OsHV-1 and *Vibrio aestuarianus* infections. *Autophagy* 11**,** 516-526.

Morga, B., Arzul, I., Chollet, B., and Renault, T. (2009). Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*. *Fish & Shellfish Immunology* 26**,** 836-842.

Morga, B., Faury, N., Guesdon, S., Chollet, B., and Renault, T. (2017). Haemocytes from *Crassostrea gigas* and OsHV-1: A promising in vitro system to study host/virus interactions. *Journal of Invertebrate Pathology* 150**,** 45-53.

Mori, K. (1979). Effects of artificial eutrophication on the metabolism of the Japanese oyster *Crassostrea gigas*. *Marine Biology* 53**,** 361-369.

Mortensen, S., Strand, A., Bodvin, T., Alfjorden, A., Skar, C.K., Jelmert, A., Aspán, A., Sælemyr, L., Naustvoll, L.J., and Albretsen, J. (2016). Summer mortalities and detection of ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. *Diseases of Aquatic Organisms* 117**,** 171-176.

Nagpal, M.L., Fox, K.F., and Fox, A. (1998). Utility of 16S–23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? *Journal of Microbiological Methods* 33**,** 211-219.

Nell, J.A., and 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**,** 39-44.

Nell, J.A., and 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 Research* 37**,** 693-700.

Nell, J.A., Smith, R.I., and Mcphee, C.C. (2000). The Sydney rock oyster *Saccostrea glomerata* (Gould 1850) breeding programme: Progress and goals. *Aquaculture Research* 31**,** 45-49.

Neuwirth, E. (2014). RColorBrewer: ColorBrewer Palettes.

Newton, R.J., Vandewalle, J.L., Borchardt, M.A., Gorelick, M.H., and Mclellan, S.L. (2011). *Lachnospiraceae* and *Bacteroidales* Alternative Fecal Indicators Reveal Chronic Human Sewage Contamination in an Urban Harbor. *Applied and Environmental Microbiology* 77**,** 6972-6981.

Nyholm, S., and Nishiguchi, M. (2008). The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. *Vie et milieu (Paris, France: 1980)* 58**,** 175.

O'connor, W., Dove, M., and Finn, B. (2008). Sydney Rock Oysters: overcoming constraints to Commercial Scale Hatchery and Nursery Production. *NSW Department of Primary Industries*.

Oceanwatch-Australia (2010). *Wapengo Lake Oyster Growers Environmental Management System* [Online]. Australian

Government. Available: http://www.oceanwatch.org.au/wp-content/uploads/2016/08/2010_02_EMS_Wapengo_Lake_final.pdf [Accessed 07/03/2019 2019].

Oceanwatch-Australia (2017). *Case Study: 1. The Shoalhaven River Catchment* [Online]. Australian Government. Available: http://www.oceanwatch.org.au/wp-content/uploads/2016/05/CS1-Shoalhaven-Catchement.pdf [Accessed 07/03/2019 2019].

Olafsen, J.A., Mikkelsen, H.V., Glaever, H.M., and Hansen, G.H. (1993). Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. *Applied and Environmental Microbiology* 59**,** 1848-1854.

Paillard, C., Le Roux, F., and Borrego, J.J. (2004). Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquatic Living Resources* 17**,** 477-498.

Pal, D., Kaur, N., Sudan, S.K., Bisht, B., Krishnamurthi, S., and Mayilraj, S. (2018). *Acidovorax kalamii* sp. Nov., isolated from a water sample of the river Ganges. *International Journal of Systematic and Evolutionary Microbiology* 68**,** 1719-1724.

Pales Espinosa, E., Corre, E., and Allam, B. (2014). Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus*. *Int J Parasitol* 44**,** 305-317.

Pappalardo, R., and Boemare, N. (1982). An intracellular *Streptococcus*, causative agent of a slowly developing disease in the Mediterranean crab, *Carcinus mediterraneus*. *Aquaculture* 28**,** 283-292.

Park, S., Park, J.M., Won, S.M., and Yoon, J.H. (2015). *Winogradskyella crassostreae* sp. nov., isolated from an oyster (*Crassostrea gigas*). *International Journal of Systematic and Evolutionary Microbiology* 65**,** 2890-2895.

Parker, L.M., Scanes, E., O'connor, W.A., Coleman, R.A., Byrne, M., Pörtner, H.O., and Ross, P.M. (2017). Ocean acidification narrows the acute thermal and salinity tolerance of the Sydney rock oyster *Saccostrea glomerata*. *Marine Pollution Bulletin* 122**,** 263-271.

Pascual, J., Macián, M.C., Arahal, D.R., Garay, E., and Pujalte, M.J. (2010). Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S *rRNA*, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes. *International Journal of Systematic and Evolutionary Microbiology* 60**,** 154-165.

Paul-Pont, I., Dhand, N.K., and Whittington, R.J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. *Aquaculture* 412-413**,** 202-214.

Pawiro, S. (2010). Safe Management of Shellfish and Harvest Waters. *World Health Organisation (WHO)*.

Pedersen, K., Skall, H.F., Lassen-Nielsen, A.M., Bjerrum, L., and Olesen, N.J. (2009). *Photobacterium damselae* subsp. *damselae*, an emerging pathogen in Danish rainbow trout, *Oncorhynchus mykiss* (Walbaum), mariculture. *Journal of Fish Diseases* 32**,** 465-472.

Perkins, F.O. (1996). The Structure Of *Perkinsus Marinus* (Mackin, Owen And Collier, 1950) Levine, 1978 With Comments On Taxonomy And Phylogeny Of *Perkinsus* Spp. *VIMS Articles* 506.

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pépin, J.F., and Keck, N. (2012). Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. *Aquaculture Environment Interactions* 2**,** 215-237.

Pernet, F., Lupo, C., Bacher, C., and Whittington, R.J. (2016). Infectious diseases in oyster aquaculture require a new integrated approach.

Philosophical Transactions of the Royal Society B: Biological Sciences 371.

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., and 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. *Frontiers in Microbiology* 6.

Petton, B., Pernet, F., Robert, R., and Boudry, P. (2013). Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquaculture Environment Interactions* 3**,** 257-273.

Pierce, M.L., Ward, J.E., Holohan, B.A., Zhao, X., and 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**,** 97-113.

Pita, L., Rix, L., Slaby, B.M., Franke, A., and Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome* 6**,** 46.

Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., and Konstantinidis, K.T. (2014). Strengths and Limitations of 16S rRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics. *PLoS ONE* 9**,** e93827.

Preheim, S.P., Boucher, Y., Wildschutte, H., David, L.A., Veneziano, D., Alm, E.J., and Polz, M.F. (2011). Metapopulation structure of *Vibrionaceae* among coastal marine invertebrates. *Environmental Microbiology* 13**,** 265-275.

Pujalte, M.J., Ortigosa, M., Macian, M.C., and Garay, E. (1999). Aerobic and facultative anaerobic heterotrophic bacteria associated to

Mediterranean oysters and seawater. *International Microbiology* 2**,** 259-266.

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

Quayle, D.B. (1961). Denman Island disease and mortality, 1960. *Fisheries Research Board of Canada* Manuscript Report 713.

Quayle, D.B. (1988). Pacific oyster culture in British Columbia. *Canadian Bulletin of Fish Aquatic Science* 218.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology* 35**,** 833.

R_Core_Team (2017). R: A language and environment for statistical computing. *R Foundation for Statistical Computing, Vienna, Austria*.

Ragone Calvo, L.M., Calvo, G.W., and Burreson, E.M. (2003). Dual disease resistance in a selectively bred eastern oyster, *Crassostrea virginica*, strain tested in Chesapeake Bay. *Aquaculture* 220**,** 69-87.

Raguenes, G., Christen, R., Guezennec, J., Pignet, P., and Barbier, G. (1997). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *Int J Syst Bacteriol* 47**,** 989-995.

Ralph, A.E., Paul, F., and Dan, C. (1999). Extrapallial abscesses associated with chronic bacterial infections in the intensively cultured juvenile Pacific oyster *Crassostrea gigas*. *Diseases of Aquatic Organisms* 37**,** 115-120.

Renault, T., Bouquet, A.L., Maurice, J.-T., Lupo, C., and Blachier, P. (2014). Ostreid Herpesvirus 1 Infection among Pacific Oyster (*Crassostrea gigas*) Spat: Relevance of Water Temperature to Virus Replication

and Circulation Prior to the Onset of Mortality. *Applied and Environmental Microbiology* 80**,** 5419-5426.

Renault, T., Cochennec, N., Le Deuff, R.-M., and Chollet, B. (1994). Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. *Bulletin of the European Association of fish Pathologists* 14**,** 64-66.

Renault, T., Lipart, C., and Arzul, I. (2001). A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *Journal of Fish Diseases* 24**,** 369-376.

Reshef, D.N., Reshef, Y.A., Finucane, H.K., Grossman, S.R., Mcvean, G., Turnbaugh, P.J., Lander, E.S., Mitzenmacher, M., and Sabeti, P.C. (2011). Detecting Novel Associations in Large Data Sets. *Science* 334**,** 1518.

Richards, G.P., Bono, J.L., Watson, M.A., and Needleman, D.S. (2014). Complete Genome Sequence for the Shellfish Pathogen *Vibrio coralliilyticus* RE98 Isolated from a Shellfish Hatchery. *Genome announcements* 2**,** e01253-01214.

Richards, G.P., Watson, M.A., Needleman, D.S., Church, K.M., and Hase, C.C. (2015). Mortalities of Eastern and Pacific oyster Larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Applied and Environmental Microbiology* 81**,** 292-297.

Riisgård, H. (1988). Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. *Marine ecology progress series. Oldendorf* 45**,** 217-223.

Robert, R., Sánchez, J.L., Pérez-Parallé, L., Ponis, E., Kamermans, P., and O'mahoney, M. (2013). A glimpse on the mollusc industry in Europe. *Aquaculture Europe* 38**,** 5-11.

Roch, P. (1999). Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture* 172**,** 125-145.

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4**,** e2584.

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., and Zilber-Rosenberg, I. (2007). The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* 5**,** 355-362.

Roubal, F.R., Masel, J., and Lester, R.J.G. (1989). Studies on *marteilia sydneyi*, agent of QX disease in the sydney rock oyster, *Saccostrea commercialis* with implications for its life cycle. *Marine and Freshwater Research* 40**,** 155-167.

Roughley, T.C. (1926). An investigation of the cause of an oyster mortality on the Georges River, New South Wales, 1924-25. *Proceedings of the Linnean Society of New South Wales* 51**,** 446-491.

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., and Nichol, S. (2001). Structure and Function of South-east Australian Estuaries. *Estuarine, Coastal and Shelf Science* 53**,** 351-384.

Rubio, A., White, I., and Ford, P. (2008). The Dynamic and Distribution of Food Supplies for the Sydney rock oyster in southern NSW estuaries. *Fisheries Research & Development Corporation* Technical report 2004/224.

Rubio-Portillo, E., Yarza, P., Peñalver, C., Ramos-Esplá, A.A., and Antón, J. (2014). New insights into *Oculina patagonica* coral diseases and their associated *Vibrio spp*. communities. *ISME Journal* 8**,** 1794-1807.

Sakowski, E.G. (2015). *The microbiome of the eastern oyster, Crassostrea virginica, in health and disease.* University of Delaware.

Samain, J.F., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P.,

Nicolas, J.L., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., and Boudry, P. (2007). Genetically based resistance to summer mortality in the Pacific oyster (Crassostrea gigas) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture* 268**,** 227-243.

Saulnier, D., De Decker, S., and Haffner, P. (2009). Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. *Journal of Microbiological Methods* 77**,** 191-197.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., and 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. *Microbial Ecology* 59**,** 787-798.

Sauvage, C., Pépin, J.F., Lapègue, S., Boudry, P., and Renault, T. (2009). Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR. *Virus Research* 142**,** 181-187.

Sawabe, T., Ogura, Y., Matsumura, Y., Gao, F., Amin, A., Mino, S., Nakagawa, S., Sawabe, T., Kumar, R., Fukui, Y., Satomi, M., Matsushima, R., Thompson, F., Gomez-Gil, B., Christen, R., Maruyama, F., Kurokawa, K., and Hayashi, T. (2013). Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Frontiers in Microbiology* 4.

Schellenberg, J., Busse, H.J., Hardt, M., Schubert, P., Wilke, T., Kämpfer, P., and Glaeser, S.P. (2017). *Winogradskyella haliclonae* sp. Nov.,

isolated from a marine sponge of the genus *Haliclona*. *International Journal of Systematic and Evolutionary Microbiology* 67**,** 4902-4910.

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., and Robinson, C.J. (2009a). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* 75**,** 7537-7541.

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., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., and Weber, C.F. (2009b). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* 75**,** 7537-7541.

Schmidt, N., and Luther, M.E. (2002). ENSO impacts on salinity in Tampa Bay, Florida. *Estuaries* 25**,** 976-984.

Schmitt, P., Duperthuy, M., Montagnani, C., Bachère, E., and Destoumieux-Garzón, D. (2012a). "Immune responses in the Pacific oyster Crassostrea gigas: An overview with focus on summer mortalities," in *Oysters: Physiology, Ecological Distribution and Mortality*. Nova Science Publishers, Inc.), 227-273.

Schmitt, P., Lorgeril, J.D., Gueguen, Y., Destoumieux-Garzón, D., and Bachère, E. (2012b). Expression, tissue localization and synergy of antimicrobial peptides and proteins in the immune response of the oyster *Crassostrea gigas*. *Developmental and Comparative Immunology* 37**,** 363-370.

Schmitt, P., Rosa, R.D., Duperthuy, M., De Lorgeril, J., Bachere, E., and Destoumieux-Garzon, D. (2012c). The Antimicrobial Defense of the

Pacific Oyster, *Crassostrea gigas*. How Diversity may Compensate for Scarcity in the Regulation of Resident/Pathogenic Microflora. *Frontiers in Microbiology* 3**,** 160.

Schott, E.J., Pecher, W.T., Okafor, F., and Vasta, G.R. (2003). The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. *Experimental Parasitology* 105**,** 232-240.

Schrobback, P., Pascoe, S., and Coglan, L. (2015). History, status and future of Australia's native Sydney rock oyster industry. *Aquatic Living Resources* 27**,** 153-165.

Segarra, A., Mauduit, F., Faury, N., Trancart, S., Dégremont, L., Tourbiez, D., Haffner, P., Barbosa-Solomieu, V., Pépin, J.-F., Travers, M.-A., and Renault, T. (2014). Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. *BMC Genomics* 15**,** 1-13.

Segarra, A., Pepin, J.F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res* 153**,** 92-99.

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environmental Microbiology* 14**,** 4-12.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I., and Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* 107**,** 20051-20056.

Sharp, K.H., Pratte, Z.A., Kerwin, A.H., Rotjan, R.D., and Stewart, F.J. (2017). Season, but not symbiont state, drives microbiome structure in the temperate coral *Astrangia poculata*. *Microbiome* 5**,** 120.

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J. (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* 334**,** 670-674.

Shreiner, A.B., Kao, J.Y., and Young, V.B. (2015). The gut microbiome in health and in disease. *Current opinion in gastroenterology* 31**,** 69-75.

Siboni, N., Balaraju, V., Carney, R., Labbate, M., and Seymour, J.R. (2016). Spatiotemporal dynamics of *Vibrio* spp. within the Sydney Harbour estuary. *Frontiers in Microbiology* 7**,** 460.

Silvester, R., Alexander, D., Antony, A.C., and Hatha, M. (2017). GroEL PCR- RFLP - An efficient tool to discriminate closely related pathogenic *Vibrio* species. *Microbial Pathogenesis* 105**,** 196-200.

Simidu, U., and Tsukamoto, K. (1985). Habitat segregation and biochemical activities of marine members of the family *vibrionaceae*. *Appl Environ Microbiol* 50**,** 781-790.

Smith, I.R., Nell, J.A., and Adlard, R. (2000). The effect of growing level and growing method on winter mortality, *Mikrocytos roughleyi*, in diploid and triploid Sydney rock oysters, *Saccostrea glomerata*. *Aquaculture* 185**,** 197-205.

Soletchnik, P., Lambert, C., and Costil, K. (2005). Summer mortality of *Crassostrea gigas* (Thunberg) in relation to environmental rearing conditions. *Journal of Shellfish Research* 24**,** 197-207.

Soletchnik, P., Ropert, M., Mazurié, J., Gildas Fleury, P., and Le Coz, F. (2007). Relationships between oyster mortality patterns and environmental data from monitoring databases along the coasts of France. *Aquaculture* 271**,** 384-400.

Soniat, T.M., Klinck, J.M., Powell, E.N., and Hofmann, E.E. (2012). Understanding the Success and Failure of Oyster Populations:

Periodicities of *Perkinsus marinus*, and Oyster Recruitment, Mortality, and Size. *Journal of Shellfish Research* 31**,** 635-646.

Spiers, Z.B., Gabor, M., Fell, S.A., Carnegie, R.B., Dove, M., O'connor, W., Frances, J., Go, J., Marsh, I.B., and Jenkins, C. (2014). Longitudinal study of winter mortality disease in Sydney rock oysters *Saccostrea glomerata*. *Diseases of Aquatic Organisms* 110**,** 151-164.

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., and Muroga, K. (1998). *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. *Diseases of Aquatic Organisms* 33**,** 111-118.

Sunila, I., Karolus, J., Lang, E.P., Mroczka, M.E., and Volk, J. (2000). Transmission of the haplosporidian parasite MSX *Haplosporidium nelsoni* to the eastern oyster *Crassostrea virginica* in an upweller system. *Diseases of Aquatic Organisms* 42**,** 153-155.

Suzuki, M.T., Taylor, L.T., and Delong, E.F. (2000). Quantitative Analysis of Small-Subunit rRNA Genes in Mixed Microbial Populations via 5′-Nuclease Assays. *Applied and Environmental Microbiology* 66**,** 4605.

Svitil, A.L., Chadhain, S., Moore, J.A., and Kirchman, D.L. (1997). Chitin Degradation Proteins Produced by the Marine Bacterium *Vibrio harveyi* Growing on Different Forms of Chitin. *Applied and Environmental Microbiology* 63**,** 408-413.

Szabo, G., Preheim, S.P., Kauffman, K.M., David, L.A., Shapiro, J., Alm, E.J., and Polz, M.F. (2012). Reproducibility of *Vibrionaceae* population structure in coastal bacterioplankton. *ISME Journal* 7**,** 509.

Takahashi, K.G., Nakamura, A., and Mori, K. (2000). Inhibitory effects of ovoglobulins on bacillary necrosis in larvae of the Pacific oyster, *Crassostrea gigas*. *Journal of Invertebrate Pathology* 75**,** 212-217.

Tarnecki, A.M., Burgos, F.A., Ray, C.L., and Arias, C.R. (2017). Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *Journal of Applied Microbiology* 123**,** 2-17.

Taylor, D.L. (1983). The Black Band Disease of Atlantic Reef Corals. II. Isolation, Cultivation, and Growth of *Phormidium corallyticum*. *Marine Ecology* 4**,** 321-328.

Thoendel, M., Jeraldo, P.R., Greenwood-Quaintance, K.E., Yao, J.Z., Chia, N., Hanssen, A.D., Abdel, M.P., and Patel, R. (2016). Comparison of microbial DNA enrichment tools for metagenomic whole genome sequencing. *Journal of Microbiological Methods* 127**,** 141-145.

Thomas, J.C., Wafula, D., Chauhan, A., Green, S.J., Gragg, R., and Jagoe, C. (2014). A survey of deepwater horizon (DWH) oil-degrading bacteria from the Eastern oyster biome and its surrounding environment. *Frontiers in Microbiology* 5.

Thompson, F.L., Austin, B., and Swings, J. (2006). *The Biology of Vibrios*. American Society of Microbiology.

Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., and Polz, M.F. (2004). Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Applied and Environmental Microbiology* 70**,** 4103-4110.

Thurber, R.V., Willner-Hall, D., Rodriguez-Mueller, B., Desnues, C., Edwards, R.A., Angly, F., Dinsdale, E., Kelly, L., and Rohwer, F. (2009). Metagenomic analysis of stressed coral holobionts. *Environmental Microbiology* 11**,** 2148-2163.

Tout, J., Siboni, N., Messer, L.F., Garren, M., Stocker, R., Webster, N.S., Ralph, P.J., and Seymour, J.R. (2015). Increased seawater temperature increases the abundance and alters the structure of natural *Vibrio* populations associated with the coral *Pocillopora damicornis*. *Frontiers in Microbiology* 6.

Trabal Fernández, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Ascencio-Valle, F., and Romero, J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis, Crassostrea gigas and Crassostrea sikamea*) during commercial production. *FEMS Microbiology Ecology* 88**,** 69-83.

Trabal, N., Mazon-Suastegui, J.M., Vazquez-Juarez, R., Asencio-Valle, F., Morales-Bojorquez, E., and Romero, J. (2012). Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. *Microbial Ecology* 64**,** 555-569.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., and Gordon, J.I. (2009). A core gut microbiome in obese and lean twins. *Nature* 457**,** 480-484.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444**,** 1027-1031.

Turner, J.W., Tallman, J.J., Macias, A., Pinnell, L.J., Elledge, N.C., Nasr Azadani, D., Nilsson, W.B., Paranjpye, R.N., Armbrust, E.V., and Strom, M.S. (2018). Comparative Genomic Analysis of *Vibrio diabolicus* and Six Taxonomic Synonyms: A First Look at the Distribution and Diversity of the Expanded Species. *Frontiers in Microbiology* 9**,** 1893-1893.

Turner, S., Pryer, K.M., Miao, V.P., and Palmer, J.D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology* 46**,** 327-338.

Ueki, A., Akasaka, H., Suzuki, D., and Ueki, K. (2006). *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *International Journal of Systematic and Evolutionary Microbiology* 56**,** 39-44.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35**,** W71-W74.

Urbanczyk, H., Ast, J.C., Higgins, M.J., Carson, J., and Dunlap, P.V. (2007). Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 2823-2829.

Urbanczyk, H., Ogura, Y., and Hayashi, T. (2013). Taxonomic revision of Harveyi clade bacteria (family *Vibrionaceae*) based on analysis of whole genome sequences. *International Journal of Systematic and Evolutionary Microbiology* 63**,** 2742-2751.

Urdaci, M.C., Stal, L.J., and Marchand, M. (1988). Occurrence of nitrogen fixation among *Vibrio* spp. *Archives of Microbiology* 150**,** 224-229.

Utami, Y.D., Kuwahara, H., Igai, K., Murakami, T., Sugaya, K., Morikawa, T., Nagura, Y., Yuki, M., Deevong, P., Inoue, T., Kihara, K., Lo, N., Yamada, A., Ohkuma, M., and Hongoh, Y. (2019). Genome analyses of uncultured TG2/ZB3 bacteria in '*Margulisbacteria*' specifically attached to ectosymbiotic spirochetes of protists in the termite gut. *ISME Journal* 13**,** 455-467.

Valdenegro-Vega, V., Naeem, S., Carson, J., Bowman, J.P., Tejedor Del Real, J.L., and Nowak, B. (2013). Culturable microbiota of ranched

southern bluefin tuna (*Thunnus maccoyii* Castelnau). *Journal of Applied Microbiology* 115**,** 923-932.

Vannini, C., Petroni, G., Verni, F., and Rosati, G. (2005). *Polynucleobacter* bacteria in the brackish-water species *Euplotes harpa* (Ciliata Hypotrichia). *Journal Eukaryotic Microbiology* 52**,** 116-122.

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P.C., Colwell, R.R., Höfle, M.G., and Pruzzo, C. (2011). Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. *ISME Journal* 6**,** 21.

Vezzulli, L., Grande, C., Reid, P.C., Hélaouët, P., Edwards, M., Höfle, M.G., Brettar, I., Colwell, R.R., and Pruzzo, C. (2016). Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. *Proceedings of the National Academy of Sciences* 113**,** E5062-E5071.

Vezzulli, L., Pezzati, E., Stauder, M., Stagnaro, L., Venier, P., and Pruzzo, C. (2015). Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. *Environmental Microbiology* 17**,** 1065-1080.

Vezzulli, L., Pruzzo, C., Huq, A., and Colwell, R.R. (2010). Environmental reservoirs of *Vibrio cholerae* and their role in cholera. *Environmental Microbiology Reports* 2**,** 27-33.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., and Pruzzo, C. (2017). Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology*.

Vijayan, N., Lema, K.A., Nedved, B.T., and Hadfield, M.G. (2019). Microbiomes of the polychaete *Hydroides elegans* (Polychaeta: Serpulidae) across its life-history stages. *Marine Biology* 166**,** 19.

Virvilis, C., and Angelidis, P. (2006). Presence of the parasite *Marteilia* sp. in the flat oyster (*Ostrea edulis* L) in Greece. *Aquaculture* 259**,** 1-5.

Waechter, M., Le Roux, F., Nicolas, J.L., Marissal, E., and Berthe, F. (2002). Characterisation of *Crassostrea gigas* spat pathogenic bacteria. *Comptes Rendus - Biologies* 325**,** 231-238.

Waldbusser, G.G., Steenson, R.A., and Green, M.A. (2011a). Oyster shell dissolution rates in estuarine waters: Effects of ph and shell legacy. *Journal of Shellfish Research* 30**,** 659-669.

Waldbusser, G.G., Voigt, E.P., Bergschneider, H., Green, M.A., and Newell, R.I.E. (2011b). Biocalcification in the Eastern Oyster (*Crassostrea virginica*) in Relation to Long-term Trends in Chesapeake Bay pH. *Estuaries and Coasts* 34**,** 221-231.

Wallace, R.K. (2001). Cultivating the Eastern Oyster, *Crassostrea virginica*. *USDA Southern Region Aquaculture Center (SRAC)* Publication No. 432.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73**,** 5261-5267.

Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., and Venables, B. (2016). gplots: Various R Programming Tools for Plotting Data. R package version 3.0.1.

Watermann, B.T., Herlyn, M., Daehne, B., Bergmann, S., Meemken, M., and Kolodzey, H. (2008). Pathology and mass mortality of Pacific oysters, C*rassostrea gigas* (Thunberg), in 2005 at the East Frisian coast, Germany. *Journal of Fish Diseases* 31**,** 621-630.

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

Wei, D.Q., Yu, T.T., Yao, J.C., Zhou, E.M., Song, Z.Q., Yin, Y.R., Ming, H., Tang, S.K., and Li, W.J. (2012). *Lysobacter thermophilus* sp. nov., isolated from a geothermal soil sample in Tengchong, south-west China. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 102**,** 643-651.

Wendling, C.C., Batista, F.M., and Wegner, K.M. (2014). Persistence, Seasonal Dynamics and Pathogenic Potential of *Vibrio* Communities from Pacific Oyster Hemolymph. *PLoS ONE* 9**,** e94256.

Wesche, S.J., Adlard, R.D., and Lester, R.J.G. (1999). Survival of spores of the oyster pathogen *Marteilia sydneyi* (Protozoa, Paramyxea) as assessed using fluorogenic dyes. *Diseases of Aquatic Organisms* 36**,** 221-226.

Whittington, R.J., Dhand, N.K., Evans, O., and Paul-Pont, I. (2015). Further observations on the influence of husbandry practices on OsHV-1 µVar mortality in Pacific oysters *Crassostrea gigas*: Age, cultivation structures and growing height. *Aquaculture* 438**,** 82-97.

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'connor, W., Booth, M., Fielder, S., and Egan, S. (2019). Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). *Microbial Biotechnology* 12**,** 275-288.

Wilson, M.E. (1995). Infectious diseases: an ecological perspective. *BMJ* 311**,** 1681-1684.

Woo, S., Yang, S.-H., Chen, H.-J., Tseng, Y.-F., Hwang, S.-J., De Palmas, S., Denis, V., Imahara, Y., Iwase, F., Yum, S., and Tang, S.-L. (2017). Geographical variations in bacterial communities associated with soft

coral *Scleronephthya gracillimum*. *PLoS ONE* 12**,** e0183663-e0183663.

Yong, L., Guanpin, Y., Hualei, W., Jixiang, C., Xianming, S., Guiwei, Z., Qiwei, W., and Xiuqin, S. (2006). Design of *Vibrio* 16S rRNA gene specific primers and their application in the analysis of seawater *Vibrio* community. *Journal of Ocean University of China* 5**,** 157-164.

Yoo, S.H., Weon, H.Y., Kim, B.Y., Kim, J.H., Baek, Y.K., Kwon, S.W., Go, S.J., and Stackebrandt, E. (2007). *Pseudoxanthomonas yeongjuensis* sp. nov., isolated from soil cultivated with Korean ginseng. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 646-649.

Yoshizawa, S., Wada, M., Kita-Tsukamoto, K., Ikemoto, E., Yokota, A., and Kogure, K. (2009). *Vibrio azureus* sp. nov., a luminous marine bacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology* 59**,** 1645-1649.

Young, C.C., Ho, M.J., Arun, A.B., Chen, W.M., Lai, W.A., Shen, F.T., Rekha, P.D., and Yassin, A.F. (2007). *Pseudoxanthomonas spadix* sp. nov., isolated from oil-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 1823-1827.

Yu, T.T., Zhou, E.M., Yin, Y.R., Yao, J.C., Ming, H., Dong, L., Li, S., Nie, G.X., and Li, W.J. (2013). *Vulcaniibacterium tengchongense* gen. nov., sp. nov. isolated from a geothermally heated soil sample, and reclassification of *Lysobacter thermophilus* Wei et al. 2012 as *Vulcaniibacterium thermophilum* comb. nov. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 104**,** 369-376.

Appendix

Marine Environmental Research 143 (2019) 124–140

Contents lists available at ScienceDirect

Marine Environmental Research

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

Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment

William L. King[a,b], Cheryl Jenkins[c], Justin R. Seymour[b], Maurizio Labbate[a,*]

[a] The School of Life Sciences, University of Technology Sydney, NSW, Australia
[b] Climate Change Cluster, University of Technology Sydney, NSW, Australia
[c] Elizabeth Macarthur Institute, New South Wales Department of Primary Industries, Menangle, NSW, Australia

ARTICLE INFO

Keywords:
Aquaculture
Climate change
Pathogen
Oyster disease
Microbiome

ABSTRACT

Shifting environmental conditions are known to be important triggers of oyster diseases. The mechanism(s) behind these synergistic effects (interplay between host, environment and pathogen/s) are often not clear, although there is evidence that shifts in environmental conditions can affect oyster immunity, and pathogen growth and virulence. However, the impact of shifting environmental parameters on the oyster microbiome and how this affects oyster health and susceptibility to infectious pathogens remains understudied. In this review, we summarise the major diseases afflicting oysters with a focus on the role of environmental factors that can catalyse or amplify disease outbreaks. We also consider the potential role of the oyster microbiome in buffering or augmenting oyster disease outbreaks and suggest that a deeper understanding of the oyster microbiome, its links to the environment and its effect on oyster health and disease susceptibility, is required to develop new frameworks for the prevention and management of oyster diseases.

1. Introduction

Oysters are filter-feeding bivalve molluscs that inhabit estuarine and coastal environments. They encompass a number of different species, many of which are heavily farmed for human consumption, supporting valuable aquaculture industries. In 2005, global bivalve aquaculture was responsible for 13.6 million metric tons of production, valued at $1.82 billion USD, with oysters responsible for 4.8 million metric tons of production (Pawiro, 2010). Four oyster species, namely, *Crassostrea gigas* (the Pacific oyster), *Saccostrea glomerata* (formerly *S. commercialis* and also known as the Sydney rock oyster), *Ostrea edulis* (the European flat oyster) and *Crassostrea virginica* (the Eastern oyster or American cupped oyster) are amongst the most heavily cultivated historically and/or currently across different regions of the world.

Infectious diseases have become a major obstacle for the successful growth and sustainability of oyster aquaculture industries, with a range of diseases having severe detrimental effects on oyster yields. For example, historical outbreaks of *C. virginica* diseases contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). While diseases of *S. glomerata* in Australia, and *O. edulis* in Europe, have also severely diminished their production capacity (René Robert et al., 2013; Schrobback et al., 2015; FAO, 2016c). Another species of oyster, *Crassostrea angulata*, was extensively cultivated in France prior to the 1970's before the industry was completely wiped out as a consequence of infectious disease outbreaks, resulting in this species being replaced by imported *C. gigas* (Roch, 1999). These few examples highlight just some of the impacts that infectious diseases have had on global oyster cultivation.

Since oysters are typically reared in uncontrolled and often dynamic coastal and estuarine environments, it is often difficult to predict, manage and control infectious disease outbreaks. Management strategies designed to control the spread of pathogens are further constrained by the ability of marine pathogens to rapidly spread over large distances, due to reduced dispersion barriers in aquatic habitats relative to terrestrial environments (Mccallum et al., 2003). Increasing evidence is showing that oyster diseases have strong environmental drivers such as temperature. Notably, outbreaks are often more severe closer to the tropics (Leung and Bates, 2013) likely due to the preference of many pathogens to grow in warmer waters (Leung and Bates, 2013), or the exertion of temperature stress as oysters reach their thermal limits (Bougrier et al., 1995). Within the context of temperature driven disease outbreaks, the implications of climate change (i.e. warming waters in non-tropical areas) on pathogen spread, transmission and virulence are a concern for future food security (Harvell et al., 2002). Specific examples supporting this concern include warming oceans driving the geographic spread of *Perkinus marinus*, the parasite responsible for

* Corresponding author. University of Technology Sydney, PO Box 123, Broadway, NSW, 2007, Australia.
E-mail address: maurizio.labbate@uts.edu.au (M. Labbate).

https://doi.org/10.1016/j.marenvres.2018.11.007
Received 8 June 2018; Received in revised form 20 October 2018; Accepted 15 November 2018
Available online 16 November 2018
0141-1136/

dermo disease in *C. virginica* (Ford, 1996; Cook et al., 1998) and, the enhanced replication and transmission of the *C. gigas* disease-causing herpesvirus OsHV-1 and growth of *Vibrio* species in *C. gigas* tissues at warmer temperatures (Petton et al., 2013; Renault et al., 2014).

The disease process has traditionally been viewed as a 'one pathogen one disease' system, a classical view pioneered by Robert Koch now known as Koch's postulates (Koch, 1884; Löffler, 1884). Since that time, our understanding of infectious disease processes has evolved from a 'classical view' to one of an 'ecological view', in which multiple factors contribute to or amplify the disease process (Wilson, 1995). As with most infectious processes, many oyster diseases appear to be complex and often proceed as a result of a shift or fracture in the interplay between environmental (e.g. temperature, salinity, pH, nutrients) and biological factors, including oyster fitness, the oyster microbiome, the abundance and virulence of external pathogens and their potential vectors (e.g. phytoplankton). Detangling the causative mechanisms of disease from this complex "interactome" (the suite of biotic and abiotic factors that participate in disease processes) is not trivial – in particular, little information is known regarding the role of the microbiome in disease protection or susceptibility. In order to develop more effective strategies for managing infectious outbreaks within oyster harvesting practices, a new understanding of the interactome and the role of the microbiome is necessary. In this review, the major diseases affecting oyster aquaculture will be covered and in particular, the potential synergistic importance of the oyster microbiome and local environmental parameters in these infectious outbreaks will be evaluated.

2. The oyster life cycle, anatomy and distribution

In this section, we will focus on four major commercial oyster species, including *C. gigas*, *S. glomerata*, *O. edulis* and *C. virginica*, which are harvested in a number of regions across the globe (Fig. 1). *C. gigas* is the most widely grown species, with commercial industries in the USA, Canada, Mexico, Chile, Argentina, South Africa, Namibia, China, Japan, Australia and a number of European countries, in particular France (FAO, 2016a). *C. virginica* is grown exclusively in the USA, Canada and Mexico (FAO, 2016b), while *S. glomerata* is only grown in Australia (FAO, 2016d). The limited production of *O. edulis* is restricted to several European nations, the USA, and South Africa (FAO, 2016c).

There are numerous microbial and viral diseases that can infect one or more stages of the oyster life cycle. Across all species of oysters, the general oyster life cycle is relatively consistent (Fig. 2). The life cycle begins with spawning, which is dependent on temperature and location (Fujiya, 1970; Wallace, 2001; FAO, 2016a; c; d). Following spawning events, fertilisation occurs, resulting in the development of a free-swimming planktonic larva (trochophore) (Wallace, 2001). At this stage, the oyster larvae are particularly vulnerable to infection by mostly viral and bacterial pathogens (Hine et al., 1992; Luna-González et al., 2002; Elston et al., 2008). After settlement on a hard surface, metamorphosis occurs developing into a juvenile oyster form called spat (Wallace, 2001). Similar to the larval form, spat are prone to infection by bacterial and viral pathogens (Waechter et al., 2002; Friedman et al., 2005). After 12–40 months of growth, the spat grows into a commercially harvestable adult oyster. Relative to the earlier forms, adult oysters are more resistant to viral infection (Dégremont, 2013) with infections from protozoan parasites more likely (Friedman and Perkins, 1994; Green and Barnes, 2010).

The oyster possesses a number of specialised tissues and organs to help it survive in its environment (Fig. 2). The gills draw in water and directs the collected food particles (such as phytoplankton) to the palps, which sort the food particles before they enter the digestive system. The digestive gland is a common site for protozoan parasite infection often culminating in oyster starvation (Alderman, 1979; Ewart and Ford,

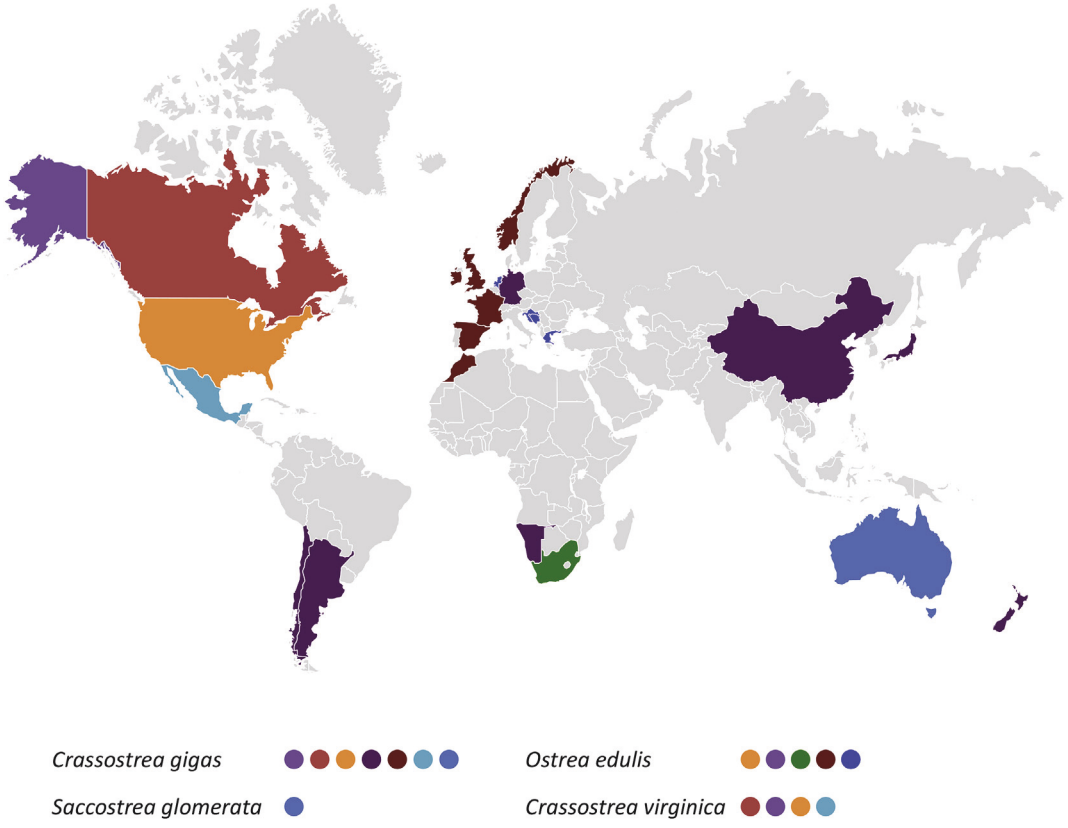


Fig. 1. Global cultivation of four oyster species. *C. gigas* is grown in the greatest number of countries, spanning North and South America, Western Europe and Australia. While *S. glomerata* is only grown in Australia. *C. virginica* is exclusively grown in North America, whereas *O. edulis* is grown in the USA, a number of European countries and South Africa.

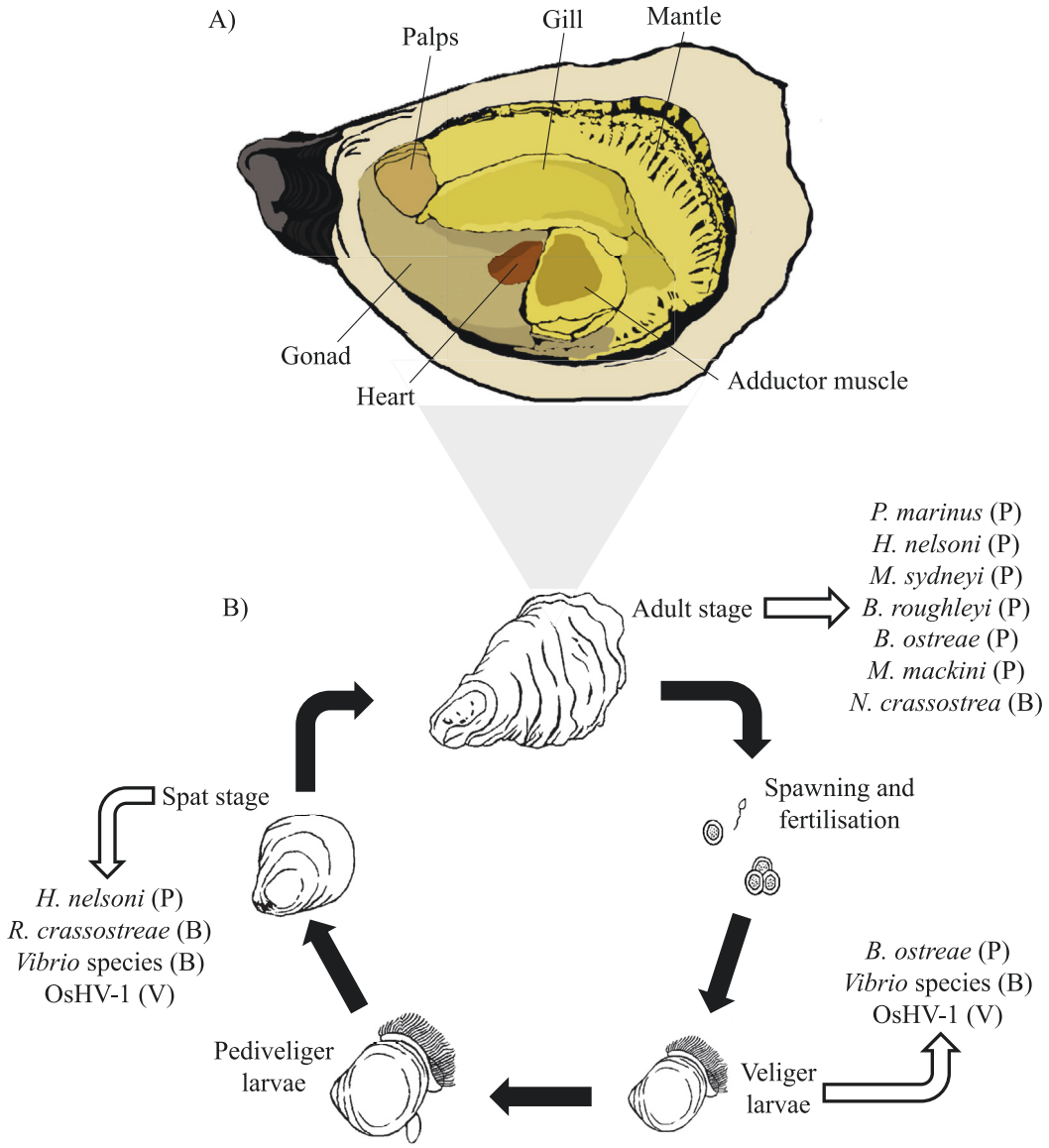


Fig. 2. The basic anatomy A) and generalised life cycle of oysters B). Oyster pathogens infect various stages of the oyster life cycle. Bacterial and viral pathogens typically infect the spat and larval stages, while the protozoan parasites dominantly infect the adult stages. Black arrows depict the life cycle progression. Black hollow arrows highlight the known pathogens of commercial oysters at each life stage. (P), (B), and (V) represent parasites, bacteria, and viral agents respectively. Image produced by Sarah J Iwanoczko

1993; Kleeman et al., 2002). The mantle acts as a sensory organ to initiate opening and closing of the shell, and forms the oyster's shell (Quayle, 1988; FAO, 2016e). Shell infections are observed from some bacterial species, resulting in mantle lesions and abnormal shell deposits (Bricelj et al., 1992). The heart is responsible for circulating the oyster hemolymph, a clear fluid that acts as the oyster 'blood' and contains cells called hemocytes with immune functions (Bachere et al., 1991). Previous research has indicated that viral pathogens are able to invade and replicate within these hemocytes (Morga et al., 2017). Finally, the gonad represents the reproductive system, which involves the production and release of gametes (spawning) (FAO, 2016e).

3. Oyster immunology

Oysters are filter feeders, filtering around 163 L per day (Riisgård, 1988) and given that the average litre of seawater contains more than a billion microbes, oysters are constantly exposed to a large number of microorganisms present in seawater. In order to combat pathogenic microorganisms, the innate immune system of the oyster is its primary defence (Schmitt et al., 2012a). This immunity is primarily facilitated by hemocytes (Fig. 3), and molecules/proteins contained in both the hemolymph and epithelial mucus secretions (Cheng and Rodrick, 1975; Itoh and Takahashi, 2008; Pales Espinosa et al., 2014; Allam and Pales Espinosa, 2016).

The oyster hemolymph is not sterile, with low concentrations (10^2-10^5 cells mL^{-1}) of bacteria, primarily from the genera *Vibrio, Pseudomonas, Aeromonas* and *Alteromonas,* which appear to naturally reside within the oyster circulatory system (Olafsen et al., 1993; Garnier et al., 2007). This raises the questions of how hemocytes differentiate between pathogens and "natural" inhabitants and may be related to the function of pattern recognition receptor proteins (e.g. peptidoglycan recognition proteins) and antimicrobial peptides (AMPs) produced by these cells. Pattern recognition receptors are produced by oyster epithelial cells and hemocytes (Itoh and Takahashi, 2008) and when stimulated (by microbial products such as peptidoglycan), activate hemocytes, allowing them to migrate to the invasion site and

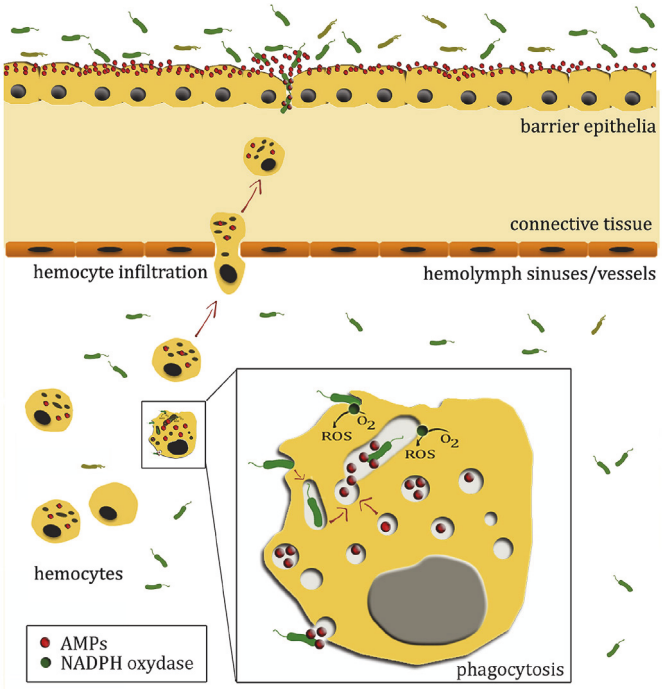


Fig. 3. An overview of the oyster cellular immune response (Schmitt et al., 2012c), published by Frontiers in Microbiology. Invading pathogens must first bypass the epithelial layer, which produces antimicrobial peptides (AMP; red circle). Following this, the circulating hemocytes in the hemolymph engulf the microbial pathogens. They are then exposed to reactive oxygen species (ROS), which are produced by either NADPH oxidase (green circle) or the mitochondria, and antimicrobial proteins such as lysozyme and AMPs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

express AMPs for a rapid and effective defence against invading microbes (Schmitt et al., 2012b). Additionally, the epithelial layer constitutively expresses a number of AMPs to further reduce microbial loads (Schmitt et al., 2012b).

Pathogens bypassing these initial defence strategies face phagocytosis by the circulating hemocytes in the hemolymph. Phagocytised pathogens (Canesi et al., 2002) are subsequently exposed to reactive oxygen species (ROS), enzymes and AMPs within the hemocyte (Labreuche et al., 2006a; Schmitt et al., 2012b). However, some bacterial and protozoan parasites are able to subvert intracellular degradation, effectively evading the oyster immune response (Schmitt et al., 2012c). This is primarily facilitated by the suppression of (ROS) generation, or reduced phagocytosis by the hemocytes (Schott et al., 2003; Labreuche et al., 2006b).

4. Diseases affecting oysters of economic importance

There are a number of well-characterised microbial diseases affecting several different oyster species. A summary of the known oyster diseases for each species is provided in Table 1.

4.1. Parasitic aetiological agents

Parasitic disease outbreaks have historically led to catastrophic losses of oysters, and large economic impacts. Dermo (also known as perkinsosis) and MSX are caused by the protozoan parasites *Perkinsus marinus*, and *Haplosporidium nelsoni* respectively (Mackin et al., 1950; Haskin et al., 1966). Specifically, historical outbreaks of dermo affecting *C. virginica* have contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). Both dermo and MSX are responsible for extensive annual mortality outbreaks, particularly along the east coast of America (Encomio et al., 2005). For *S. glomerata*, Queensland unknown disease (QX) is caused by the protozoan parasite, *Marteilia sydneyi* (Anderson et al., 1994; Kleeman et al., 2002), while the aetiolgical agent of *S. glomerata* winter mortality is unclear with conflicting morphological, histological and molecular evidence from different laboratories (Carnegie et al., 2014; Spiers et al., 2014). These two diseases have reduced cultivation in some Australian estuaries by as much as 97% (Nell and Perkins, 2006; O'Connor et al., 2008; Dove et al., 2013b). QX disease has been particularly harsh with mortality rates as high as 85–95% (Anderson et al., 1994; Bezemer et al., 2006). The decline of the *O. edulis* industry in Europe has been attributed to two parasitic diseases, marteiliosis (also known as Aber disease) and bonamiasis (René Robert et al., 2013), caused by *Bonamia ostreae* and *Marteilia refringens* respectively (Alderman, 1979; Balouet et al., 1983; Elston et al., 1986).

4.1.1. Disease process of parasites

Parasitic diseases are chronic, typically taking weeks or months to kill their host through disruption of different tissue(s) usually causing effects such as oyster starvation, and/or tissue lysis (Andrews and Hewatt, 1957; Haskin et al., 1966; Balouet et al., 1983; Adlard and Ernst, 1995; Hervio et al., 1996). This section will review what is known about parasitic infections of oysters including the oyster tissue (s) where infection is initiated, the process(es) by which parasites move to other tissues/sites in the oyster and, process(es) that lead to oyster death.

Of the various oyster parasites, the point/site of infection can vary and include the gill and palps for *M. sydneyi* (Kleeman et al., 2002), and the mantle epithelium for *P. marinus* (Allam et al., 2013). However, for the remaining oyster parasites (*H. nelsoni*, *M. refringens*, *B. ostreae*, and *M. mackini*), the site(s) of infection are unknown and is an area that requires additional research. Despite this, gill infections are commonly observed for these parasites (Haskin et al., 1966; Balouet et al., 1983; Farley et al., 1988; Kleeman et al., 2002; Ragone Calvo et al., 2003; Carnegie and Burreson, 2011), indicating that oyster filter feeding is an important process for the transmission of the parasite into the oyster with the gills possibly acting as the point of infection.

Following initial infection, subsequent dissemination to specific tissues or cells varies depending on the infecting parasite, with hemocytes, the digestive gland and connective tissue known targets. *P. marinus* and *B. ostreae* are phagocytosed by the circulating hemocytes (Balouet et al., 1983; Schott et al., 2003), and are both able to survive the process through degradation or preventing the formation of toxic reactive oxygen species inside the hemocyte (Schott et al., 2003; Morga et al., 2009). These parasites are able to proliferate within the hemocyte and use them as a vehicle to spread throughout the oyster (Montes et al., 1994; Perkins, 1996), resulting in the lysis of various host tissues and/or blockage of the oyster circulatory system thus culminating in mortality (Andrews and Hewatt, 1957; Balouet et al., 1983; Choi et al., 1989; Encomio et al., 2005). For the two *Marteilia* parasites, *M. sydneyi* and *M. refringens,* both lead to an infection of the digestive gland resulting in disrupted growth and impaired nutrient uptake leading to oyster starvation and mortality (Alderman, 1979; Camacho et al., 1997; Kleeman et al., 2002; Green et al., 2011). Destruction of the digestive gland and tubules is also observed for oysters infected with *H. nelsoni* (Ford and Haskin, 1982), but it is not clear whether the parasite also affects nutrient uptake similar to the *Marteilia* parasites. While it is known that systemic dissemination of *M. sydneyi* cells follows on from the initial gill and palp infection (Kleeman et al., 2002), it is unclear whether *M. refringens* and *H. nelsoni* also disseminate towards the digestive gland/tubules from an initial infection site, or whether the infection is initiated in the digestive gland/tubules. Connective tissue cells (cells between organ tissues) of the oyster are infected by *M. mackini* causing mortality through tissue disruption and necrosis (Hervio et al., 1996; Bower et al., 1997). This process produces characteristic green pustules, ulcers and abscesses on several different

Table 1
Diseases of economically important oyster species, their affected life stage and the pathology seen for each disease.

Oyster species	Disease/pathogen (agent)	Affected oyster stage	Pathology	Geographical distribution	Mortality range (%)	References
The Eastern oyster (*Crassostrea virginica*)	Dermo/*Perkinsus marinus* (Protozoan)	Adult	Tissue lysis, blockage of circulatory system	USA East Coast	20–85	Andrews and Hewatt, 1957; Ford, 1996
	MSX/*Haplosporidium nelsoni* (Protozoan)	Spat and adult	Epithelium infection, respiratory and digestive impacts	USA East Coast	33–95	Haskin et al., 1966; Ford and Haskin, 1982; Ewart and Ford, 1993
	ROD/*Roseovarius crassostreae* (Bacterium)	Spat	Mantle lesions, conchiolin deposits, tissue degradation	USA East Coast	54–75	Bricelj et al., 1992; Boardman et al., 2008
Sydney rock oyster (*Saccostrea glomerata*)	QX/*Marteilia sydneyi* (Protozoan)	Adult	Digestive tubule destruction, starvation	Australian East Coast	22–99	Kleeman et al., 2002; Nell and Perkins, 2006
	Winter Mortality/*Bonamia roughleyi* † (Protozoan)	Adult	Connective tissue disruption, ulcers, impaired muscle contractions, necrotic tissues	Australian East Coast	9–52	Roughley, 1926; Mackin, 1959; Farley et al., 1988; Smith et al., 2000
European flat oyster (*Ostrea edulis*)	Marteiliosis/*Marteilia refringens* (Protozoan)	‡	Digestive gland infection, impaired growth, starvation	France, Spain, Portugal and Greece	50–90	Alderman, 1979; Virvilis and Angelidis, 2006; Bower, 2011; López-Sanmartín et al., 2015
	Bonamiasis/*Bonamia ostreae* (Protozoan)	Adult, larvae	Gill and mantle lesions, parasite resides within hemocytes	France, Spain, England, Denmark, the Netherlands, USA West Coast	40–80	Balouet et al., 1983; Elston et al., 1986
Pacific Oyster (*Crassostrea gigas*)	Denman Island disease/*Mikrocytos mackini* (Protozoan)	Adult	Green pustules, ulcers and abscesses on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	17–53	Quayle, 1961; Farley et al., 1988; Elston et al., 2015
	Nocardiosis/*Nocardia crassostreae* (Bacterium)	Adult	Green pustules and lesions on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	47–50	Friedman et al. (1991)
	Vibriosis (Bacillary necrosis)/*Vibrio* spp. (Bacterium)	Larvae, spat	Abnormal swimming, necrosis, lesions	Worldwide	76–100*	Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002; Elston et al., 2008
	Pacific Oyster Mortality Syndrome/ OsHV-1 and OsHV-1 µ variant (Virus)	Larvae, spat	Lesions and cells with viral inclusions and hypertrophied nuclei. Reduced feeding and impaired swimming in larvae	USA East Coast, Australia, New Zealand, France, Sweden and Norway	40–100	Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016
	Summer Mortality/Unknown or multifactorial§	All stages	ill defined, characterised by high level mortalities during the warmer months	USA, France, Australia, Japan, Germany, Ireland, Sweden and Norway	30–100	Mori, 1979; Soletchnik et al., 2005; Burge et al., 2007; Garnier et al., 2007; Malham et al., 2009

†The aetiological agent of winter mortality may not be *Bonamia roughleyi*.
‡Age not reported, likely adult oysters are affected by marteiliosis as seen in QX disease.
§While no definite aetiological agent has been found, OsHV-1 and a number of *Vibrio* spp. have been associated with this disease usually during periods of host-stress (e.g. reproductive or heat stress).
*Depending on the *Vibrio* strain and bacterial concentration used.

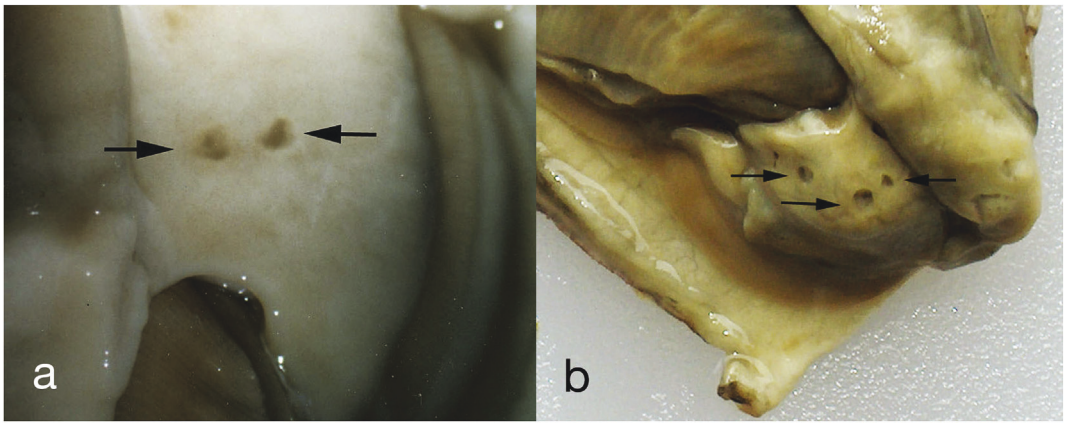


Fig. 4. Ulcerated lesions (black arrows) on the labial palps of *Crassostrea gigas* characteristic of Denman Island Disease (Elston et al., 2015), published by Diseases of Aquatic Organisms, © Inter-Research 2015.

oyster tissues (Fig. 4) (Farley et al., 1988; Hervio et al., 1996).

Since the aetiological agent(s) of winter mortality is still being debated (Spiers et al., 2014), the disease process remains poorly understood. Spiers et al. (2014) carried out a longitudinal study with the aim of determining the aetiological agent of winter mortality. While the presence of a *Bonamia* spp. was confirmed by PCR, the occurrence of this parasitic organism was quite low (3% of all samples) and the 18S rRNA sequence of the observed protozoan was closely related to another organism, *B. exitiosa* which has previously been identified in *S. glomerata* (Carnegie et al., 2014) but not in association with clinical disease. The low prevalence of *Bonamia* spp. DNA in the Spiers et al. study was inconsistent with the high prevalence of pathological observations. Similarly, no *Bonamia* spp. was found within the lesions of the oysters (Spiers et al., 2014). While this research suggests that another organism may be causing or perhaps working with *Bonamia* spp. in winter mortality, this study only observed a 10% total mortality over the entire study period, which is not an extensive outbreak. As a result, further studies are required to elucidate the aetiological agent(s) of winter mortality before further research on the disease process can be elucidated.

4.1.2. Environmental reservoirs and transmission of infectious parasites

For the majority of infectious parasites, the environmental reservoir and details of transmission to and between oysters is not completely understood. On reservoirs, it is unknown whether the parasite is residing in the environment (i.e. the water column or in sediments), or whether an intermediate host is acting as an environmental reservoir. It may also be possible that the parasite is using the intermediate host for maturation and then residing in another unknown organism. For example, *M. sydneyi* spores are only able to survive in the marine environment for up to 35 days, which is inconsistent with the yearly cycle of QX disease outbreaks (Wesche et al., 1999). It is therefore likely that an intermediate host exists as a reservoir of the parasite. Recent evidence suggests that *M. sydneyi* is present within the intestinal epithelium of the marine worm *Nephtys australiensis* and it has been proposed that this organism may act as a reservoir for *M. sydneyi* or may be critical for the maturation and transmission of *M. sydneyi* (Adlard and Nolan, 2015). Therefore, further research is necessary to determine where these parasites reside, and for those parasites with intermediate hosts, whether their intermediate host may act as that reservoir.

In regards to transmission, parasites can either be transmitted directly or via an intermediate. Direct transmission of parasites between infected and naïve oysters has been observed for dermo, bonamiasis, and Denman island disease (Elston et al., 1986; Quayle, 1988; Ewart and Ford, 1993; Hervio et al., 1996). While the causative agents of MSX, QX, and marteiliosis require an intermediate host(s) for the maturation and transmission of the parasite.

For those directly transmitted parasites, *P. marinus* is shed into the water column from infected oyster hosts, which can then be ingested by neighbouring oysters (Ewart and Ford, 1993). Similarly, only cohabitation with infected oysters is necessary for the transfer of *B. ostreae* and *M. mackini* to naïve hosts (Elston et al., 1986; Quayle, 1988; Hervio et al., 1996). The larvae of *O. edulis* can also be infected with *B. ostreae*, potentially allowing them to act as a reservoir of the parasite in the environment (Arzul et al., 2011).

For those parasites with no direct transmission, early laboratory-based studies were unsuccessful in transmitting *H. nelsoni* to uninfected oysters through co-incubation with infected oysters (Ewart and Ford, 1993). Later studies have demonstrated that an intermediate carrier capable of penetrating 1 mm^2 filters is required for transmission to naïve oysters (Sunila et al., 2000). Similarly, while field studies investigating the transmission of *M. refringens* into *O. edulis* demonstrated that the parasite was transmissible through cohabitation of uninfected with infected oysters or by deploying uninfected oysters in areas known to contain the pathogen (Berthe et al., 1998), laboratory-based cohabitation experiments and inoculations were insufficient to cause infections (Berthe et al., 1998). Later studies have identified two copepod species, *Paracartia grani* and *Paracartia latisetosa,* harbouring *M. refringens* and are implicated in the transmission of this parasite (Audemard et al., 2002; Arzul et al., 2014). This is similar for *M. sydneyi,* in which before an infected oyster dies, almost all of the *M. sydneyi* sporonts (Fig. 5) are shed into the environment (Roubal et al., 1989). However, direct transmission studies have been unable to transmit the parasite to naïve oysters (Lester, 1986). Likely the intermediate host, *Nephtys australiensis*, and possibly other unknown hosts, are needed to transmit *M. sydneyi* to naïve oysters (Adlard and Nolan, 2015).

4.1.3. Management strategies of parasitic diseases

Attempts to reduce the impact of these parasitic diseases revolve around the development of breeding programs, modified husbandry practices, and quarantining affected areas (Nell et al., 2000; Smith et al., 2000; Ragone Calvo et al., 2003; Green et al., 2011; Lynch et al., 2014). Of these strategies, breeding for disease-resistance has been the most successful (Ragone Calvo et al., 2003; Dove et al., 2013a, 2013b; Lynch et al., 2014). Dual resistance has been bred into *C. virginica* against dermo and MSX disease, leading to an improved survivability of approximately 30–60% when compared to control oyster stocks (Ragone Calvo et al., 2003). Similarly, a breeding programme carried out in Ireland since 1988 has successfully mitigated the damage of *B. ostreae* on *O. edulis* populations, culminating in an increased survival rate of 75% of market sized adult oysters, relative to 5–10% before the breeding programme began (Lynch et al., 2014). Breeding for disease-resistance has also been successful for *S. glomerata* against QX and winter mortality, with oyster mortality decreasing from 97% to 28% for

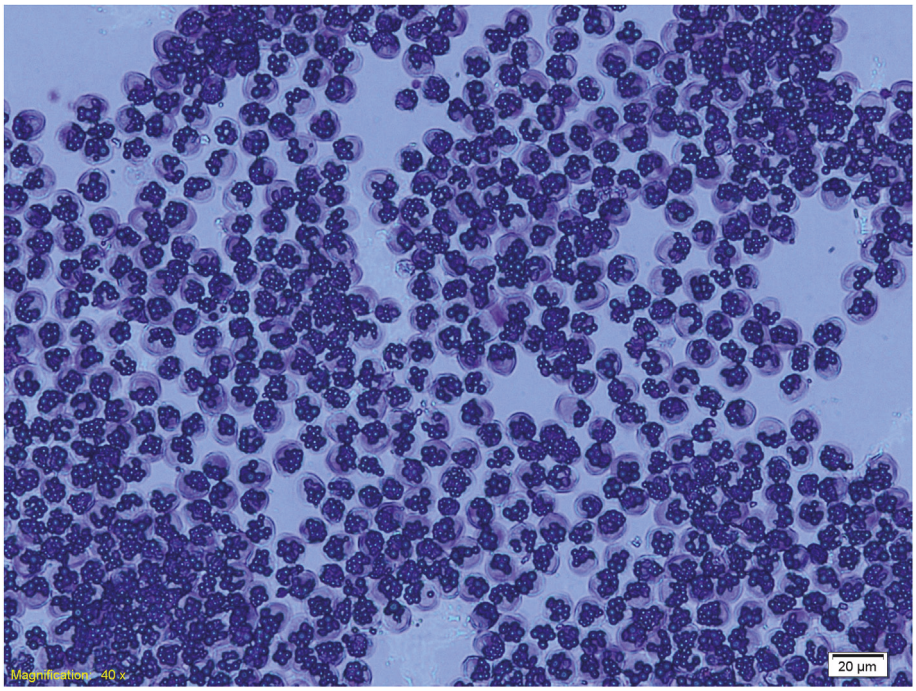


Fig. 5. Purified *Marteilia sydneyi* sporonts, the causative agent of QX disease of *Saccrostrea glomerata*. Image is at 40x magnification. Image produced by Cheryl Jenkins and Jeffrey Go at the New South Wales Department of Primary Industries.

Table 2
Vibrio pathogens of *Crassostrea gigas* and their affected life stage. Bacterial pathogens are typically isolated from diseased oysters and used in virulence assays to determine pathogenicity.

Bacterial agent	Stage affected	Reference
V. tubiashii	Larvae	Jeffries, 1982; Hada et al., 1984; Takahashi et al., 2000
V. splendidus	Larvae	Sugumar et al. (1998)
	Spat	Waechter et al. (2002)
	Adult	Garnier et al. (2007)
V. alginolyticus	Larvae	Luna-González et al. (2002)
	Adult[b]	Go et al. (2017)
V. splendidus group	Spat	Gay et al. (2004)
	Adult	Garnier et al. (2007)
V. aestuarianus	Spat	Saulnier et al., 2009, 2010
	Adult	Garnier et al., 2007; Saulnier et al., 2010
V. lentus	Spat	Saulnier et al. (2010)
V. harveyi	Spat	Saulnier et al. (2010)
	Adult[‡]	Go et al. (2017)
V. coralliilyticus	Spat	Elston et al., 2008; Richards et al., 2015
V. crassostreae	Spat[a]	Lemire et al., 2015; Bruto et al., 2017;
	Adult[b]	Go et al. (2017)

[a] Based on supplementary information for the production of specific pathogen free (SPF) oysters.
[b] Used in an inoculation cocktail comprised of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and *V. crassostreae*.

QX, and 52%–23% for winter mortality (Dove et al., 2013b). Modified husbandry practices are used to limit the exposure time of the oyster to the parasite, this can be done by altering the growing height of the oysters, or by transplanting oysters after the disease period has passed. Modified husbandry practices can be seen with winter mortality, in which *S. glomerata* are grown at a position located 15–30 cm higher in the tidal range than the typical growth height (approximately mid-tide level) (Smith et al., 2000).

4.2. Bacterial aetiological agents

4.2.1. Disease process of bacterial pathogens

Bacterial disease outbreaks are often sudden, resulting in severe mortality in a matter of days or weeks (Jeffries, 1982; Friedman and Hedrick, 1991; Bricelj et al., 1992). *Roseovarius crassostreae*, the aetiological agent of ROD in *C. virginica* causes sporadic outbreaks during the summer months, with mortalities up to 75% (Bricelj et al., 1992). For vibriosis of *C. gigas*, mortalities can exceed 90% within a period of only 24 h (Takahashi et al., 2000). While *Nocardia crassostreae* the causative agent of *C. gigas* acts slower, resulting in mortalities up to 47% over 34 days (Friedman and Hedrick, 1991).

Lesions are common symptoms for oysters affected by ROD, nocardiosis, and vibriosis, and spat are often the most at risk for infection (Jeffries, 1982; Bricelj et al., 1992; Bower, 2006). In addition, *R. crassostreae* colonises the inner shell surface of *C. virginica*; the oyster responds to this intrusion through the formation of conchiolin (organic compound secretions involved in shell formation) deposits on the shell, which is thought to act as a barrier to contain further bacterial infection (Boardman et al., 2008). Additional pathological symptoms include lesions on the mantle, degradation of muscles and tissues, infiltration of hemocytes into the epithelium of the oyster, as well as lesions under the hinge ligament (Bricelj et al., 1992). Conchiolin deposits filled with bacteria and necrotic cells are also observed in vibriosis of *C. gigas* (Ralph et al., 1999). Conversely, conchiolin deposits aren't seen in nocardiosis, instead oysters display green pustules and lesions on a number of different oyster tissues (Bower, 2006).

A number of different *Vibrio* species cause disease in *C. gigas*, resulting in either vibriosis or bacillary necrosis (Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002). A summary of the known *Vibrio* pathogens can be seen in Table 2. *C. gigas* larvae and spat are typically affected by *Vibrio* infections (Jeffries, 1982; Elston et al., 2008). Vibriosis in oyster larvae involves tissue necrosis (Fig. 6) and abnormal swimming culminating in mortality (Jeffries, 1982). Vibriosis of spat can lead to lesions and necrosis of the tissues (Elston et al., 2008). As seawater temperatures rise with climate change, the spread and growth of bacteria such as *Vibrio,* which prefer warmer waters, has been

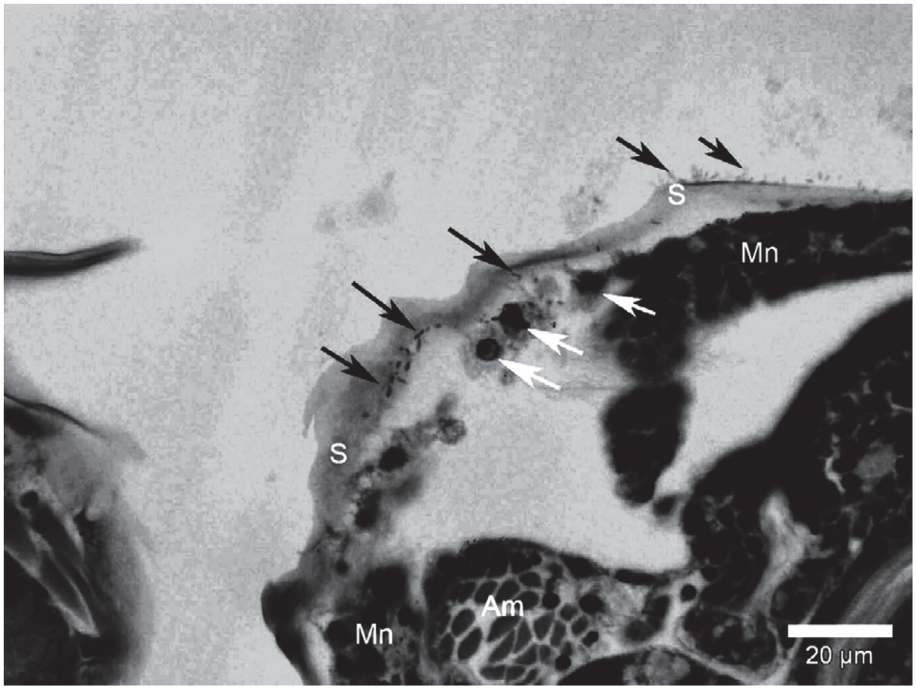


Fig. 6. Histological section of *Crassotrea gigas* larvae, with a persistent *Vibrio* infection (black arrows), as well as necrotic epithelial cells (white arrows). Larvae tissue are marked as S (shell), Mn (mantle) and Am (adductor muscle) (Elston et al., 2008). Published by Diseases of Aquatic Organisms, © Inter-Research 2008.

predicted to be enhanced (Martinez-Urtaza et al., 2010; Vezzulli et al., 2016). Notably, an elevation in surface seawater temperature was linked to the resurgence of the oyster pathogen *Vibrio coralliilyticus* on the North American Pacific Coast, where it was responsible for a major *C. gigas* mortality event (Elston et al., 2008; Richards et al., 2015).

While vibriosis tends to affect larvae and spat, experimental injections of adult oysters with *Vibrio* species, including *V. aestuarianus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Garnier et al., 2007; Saulnier et al., 2010; Go et al., 2017) has also been shown to induce mortality, with a weakening of the adductor muscle and necrotic oyster tissues observed (Garnier et al., 2007). However, the injection of bacteria into oyster hemolymph/tissues may not be a good model for the natural transmission of *Vibrio* infections in the environment. Often *Vibrio* infections, particularly from the *V. splendidus* group, are found to occur concurrently with a herpesvirus infection (OsHV-1) (Segarra et al., 2010; Pernet et al., 2012; Keeling et al., 2014; De Lorgeril et al., 2018) with a recent study highlighting a synergistic, polymicrobial infection process, in which the oyster immune system is suppressed following OsHV-1 infection, allowing for bacteraemia to occur (De Lorgeril et al., 2018).

4.2.2. Environmental reservoirs and transmission of bacterial pathogens

Often, bacterial infections are opportunistic, requiring an environmental stressor or immune suppression of the oyster host before infection occurs (Bricelj et al., 1992; De Lorgeril et al., 2018). No studies have identified environmental reservoirs for *N. crassostreae* and *R. crassostreae*, while *Vibrio* species are ubiquitous in the environment and are commonly found in the water column, sediments, vegetation, and associated with other organisms (Vezzulli et al., 2010; Chase et al., 2015). Given the worldwide distribution of vibriosis, it is possible that *Vibrio* bacteria are members of the oyster microbiome that are awaiting favourable conditions to cause disease, such as with OsHV-1 infection (De Lorgeril et al., 2018) or with the acquisition of virulence plasmids through horizontal gene transfer (Bruto et al., 2017). Whereas *N. crassostreae* and *R. crassostreae* are localised to the USA northwest coast and USA east coast respectively (Friedman et al., 1991; Bricelj et al., 1992), because of this, there likely exists an unknown seasonal environmental reservoir for these pathogens.

Regarding transmission, laboratory transmission studies of ROD indicate that *R. crassostreae* is transmissible with symptoms arising 3–7 weeks after cohabitation with infected oysters (Lewis et al., 1996). Conversely, laboratory transmission of *N. crassostreae*, has not been successful (Friedman et al., 1991) suggesting either an unknown transmission element is required to infect new oysters, or that the infection is opportunistic, requiring environmental stressors such as the high temperatures typically seen during summer months, in order to induce disease (Friedman et al., 1991). Transmission of *Vibrio* species from infected to naïve oysters is likely bacterial species dependent. While one study was able to cause vibriosis in naïve animals by cohabiting them with oysters injected with a mixture of *V. splendidus* and *V. aestuarianus* (De Decker and Saulnier, 2011), another study was unable to transmit vibriosis when using a *Vibrio* cocktail made of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Go et al., 2017) possibly contrasting a difference in experimental methodology, or a difference between the transmission of different *Vibrio* species.

4.2.3. Management strategies for bacterial pathogens

No control measures are currently employed to contain nocardiosis of *C. gigas* or for ROD of *C. virginica*. Often vibrio blooms due to favourable environmental conditions (warm water and excess nutrients) are the cause of vibriosis for larvae and spat in hatchery settings (Elston et al., 2008). Monitoring environmental conditions and water quality may help predict *Vibrio* outbreaks, possibly allowing farmers to change their water source in hatchery settings, or to remove oysters from the environment until the bloom has passed.

4.3. Viral aetiological agents

Of these economically valuable oyster species, only one virus, ostreid herpesvirus 1 (OsHV-1), has been identified as a major disease-causing pathogen (Hine et al., 1992; Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). OsHV-1 primarily infects and induces mortality in *C. gigas* larvae and spat, as well as young adult oysters, with

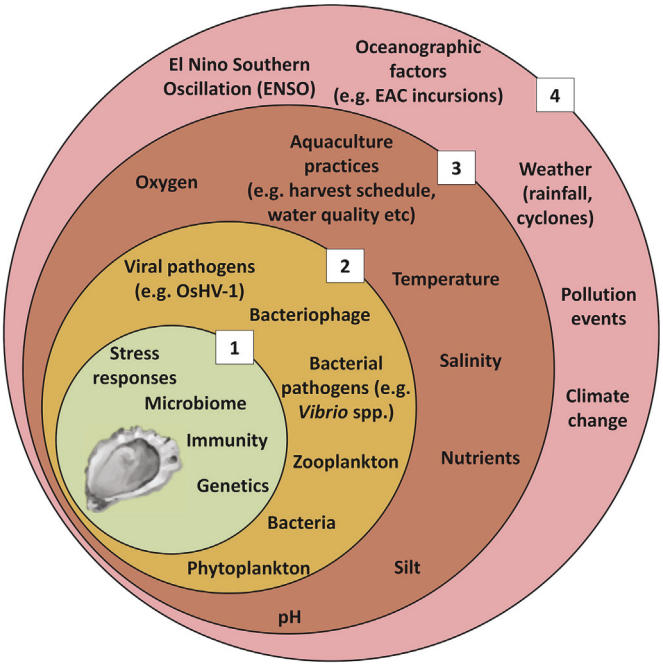


Fig. 7. The interactome/synergism of oyster diseases. The outer rings are large scale environmental events (e.g. climate change) that influence the lower rings (e.g. temperature) allowing for a cascade effect that eventually influences microbial communities and pathogens (e.g. increased pathogen proliferation), that can then act on the oyster host.

observed mortality rates ranging between 40 and 100% (Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010). OsHV-1 has been linked to a number of large mortality events across the globe and is continuing to spread (Burge et al., 2006; Segarra et al., 2010; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). Oysters infected with OsHV-1 display both lesions and cellular infections throughout the gills, mantle, digestive glands and in the hemocytes, whereby cells show altered cellular morphology, such as abnormal shapes, enlarged nuclei, nuclear fragmentation and nuclear inclusions (Hine et al., 1992; Renault et al., 1994; Friedman et al., 2005). OsHV-1 infected larvae have also been observed to have reduced feeding capacity and impaired swimming abilities (Hine et al., 1992; Renault et al., 2001).

Since its characterisation, a number of variant forms of OsHV-1 have been discovered (Arzul et al., 2001; Segarra et al., 2010; Martenot et al., 2011). Of these, a micro-variant form, named OsHV-1 µvar (Segarra et al., 2010), has been associated with mortality outbreaks in a number of countries (Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016). This micro-variant form has a number of nucleotide substitutions and deletions that distinguish it from the original variant (Segarra et al., 2010). Infection by OsHV-1 µvar acts to suppress the oyster's immune system thereby allowing opportunistic bacteria (such as *Vibrio* bacteria) to cause bacteraemia (De Lorgeril et al., 2018), and the oyster microbiome also shifts in response to viral infection (De Lorgeril et al., 2018). Furthermore, treating OsHV-1 µvar infected oysters with antibiotics significantly reduces the number of mortalities (Petton et al., 2015). As the oyster microbiome can act as a source of opportunistic pathogens (Lokmer and Wegner, 2015), further studies are required to examine the relationship (and possible interactions) between OsHV-1 µvar and the oyster microbiome.

OsHV-1 has been experimentally transferred to naïve oysters within the laboratory (Dégremont et al., 2013; Petton et al., 2015). Notably, it has also been demonstrated that OsHV-1 resistant oysters infected with OsHV-1 are unable to transmit the virus to naïve oysters, and resistant oysters maintained an overall lower viral load than non-resistant oysters (Dégremont et al., 2013). Management strategies have been focused on movement controls (quarantining affected areas) and the production of genetic lines of oysters resistant to OsHV-1, that are able to reduce viral replication and more easily recover from viral infection (Segarra et al., 2014).

4.4. Unknown aetiological agents

In recent decades a phenomenon known as 'summer mortality' has heavily impacted the *C. gigas* aquaculture industry globally. These disease outbreaks have occurred all over the world including France (Garnier et al., 2007; Segarra et al., 2010), Australia (Jenkins et al., 2013; Go et al., 2017), the USA (Friedman et al., 2005), Germany (Watermann et al., 2008), Ireland (Malham et al., 2009), Japan (Mori, 1979) and in recent years Sweden and Norway (Mortensen et al., 2016). Summer mortality is marked by the loss of over 30% of oyster stocks (Soletchnik et al., 2005, 2007) and in some instances has been observed to result in 100% mortality (Burge et al., 2007). Summer mortality has been responsible for catastrophic losses of *C. gigas* harvests since the 1960's (Mori, 1979), but the mechanisms involved and if a pathogen(s) is responsible remains largely unknown. A number of different factors have been implicated in these mortalities, including rising seawater temperatures, eutrophication, infections by *Vibrio* species and the herpesvirus OsHV-1, but often the cause appears to be multifactorial (Malham et al., 2009; Dégremont et al., 2013; Lemire et al., 2015; Petton et al., 2015), involving the interplay of multiple biotic and abiotic factors, which may affect the oyster immune system allowing opportunistic pathogens to take hold (Samain et al., 2007; Malham et al., 2009), and/or the abundance and virulence of pathogens. In this sense, summer mortality is an umbrella term that likely encompasses a number of different diseases with known or unknown aetiological agents. The bulk of recent research suggests a major role for OsHV-1 in summer mortality, with many research groups detecting this virus when disease outbreaks occur (Friedman et al., 2005; Burge et al., 2006, 2007; Segarra et al., 2010; Jenkins et al., 2013). It is notable however, that OsHV-1 was not detected in a recent summer mortality event in Australia (Go et al., 2017). Likely, periods of high temperature and low salinity acted to stress the oyster, resulting in immune suppression (Go et al., 2017), and allowing for bacterial infection to occur. This is evidenced with OsHV-1, in which infection acts to suppress the oyster's immune system allowing for bacteraemia to kill the host (De Lorgeril et al., 2018).

5. The role of the environment in facilitating disease outbreaks

The environment within which an organism resides, the pathogens to which it is exposed to, and the host's physiology (including the microbiome) can be considered an "interactome" that influences disease dynamics (Fig. 7) (Arthur et al., 2017). The concept of the interactome is particularly relevant to oysters given that they filter large quantities of water, thereby increasing the chance of exposure to pathogens. However, while there has been a substantial amount of research into the mechanisms behind diseases of oysters due to the global economic importance of these species, only recently have studies taken a more holistic approach to unravelling the interactome (Pernet et al., 2016). As a result, there is a need to move beyond viewing oyster diseases from a classical perspective (Koch's postulates; one disease one pathogen), to a more ecological viewpoint of disease.

There is growing evidence that environmental factors are critical in the spread and severity of oyster diseases (Ford, 1996; Petton et al., 2013; Mortensen et al., 2016). A summary of the environmental parameters that have been found to influence oyster diseases is presented in Table 3.

Table 3
Environmental factors that influence oyster diseases.

Disease	Influential environmental parameters	References
Dermo	Increased winter temperature Increased salinity	Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Soniat et al., 2012
MSX	Increased winter temperature Increased salinity	Haskin and Ford, 1982; Ford et al., 1999
ROD	Increased temperature Increased salinity	Lewis et al. (1996)
QX	Increased temperature Decreased salinity for spores	Wesche et al. (1999)
Winter mortality	Dry autumns Increased salinity Decreased temperature	Roughley, 1926; Butt et al., 2006; Nell and Perkins, 2006
Marteiliosis	Increased temperature	Berthe et al., 1998; Audemard et al., 2001
Bonamiasis	Decreased temperature Increased salinity Higher pH[a]	Arzul et al. (2009)
Denman Island disease	Decreased temperature	Hervio et al., 1996; Bower et al., 1997
Nocardiosis	Increased temperature Lower dissolved oxygen	Friedman et al., 1991; Engelsma et al., 2008
Vibriosis	Higher temperature to increase *Vibrio* growth Low salinity inhibits *Vibrio* infectivity	Lacoste et al., 2001; Elston et al., 2008; Richards et al., 2015
OsHV-1	Increased temperature for viral replication Increased temperature for viral transmission Rainfall	Jenkins et al., 2013; Petton et al., 2013; Renault et al., 2014
Summer mortality	Chlorophyll *a* Temperature Turbidity Salinity Nutrients (Ammonium, Phosphate, Nitrate, Nitrite, Silicate)	Soletchnik et al., 2007; Malham et al., 2009

[a] Observation made by the authors that more acidic media increased parasite mortalities.

5.1. Temperature

In marine environments, sea temperature is a major driver of oyster disease outbreaks with temperature shifts mostly dictated by the seasons, although oceanic phenomena (such as marine heat waves) can also play a role (Table 3). Warmer temperatures are known to affect the severity and prevalence of dermo, MSX, ROD, marteiliosis, QX, nocardiosis, vibriosis, OsHV-1 and summer mortality, while bonamiasis is most prominent during cooler water temperatures (Ford, 1996; Lewis et al., 1996; Wesche et al., 1999; Arzul et al., 2009; Malham et al., 2009; Green et al., 2011; Petton et al., 2013). As a result, marteiliosis, nocardiosis, summer mortality (including OsHV-1), MSX and ROD disease outbreaks occur, or are more severe, during the summer months (Friedman et al., 1991, 2005; Berthe et al., 1998; Boettcher et al., 1999; Soletchnik et al., 2007; Engelsma et al., 2008; Watermann et al., 2008), with outbreaks of vibriosis occurring during unusually warmer than normal summer temperatures (Lacoste et al., 2001; Elston et al., 2008). Where cooler temperatures would normally supress disease, there is evidence that unusually warm winters are a catalyst for increased intensity of dermo and MSX outbreaks in the following summer (Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Ford et al., 1999). It's not always clear why warmer temperatures induce disease outbreaks, but there is evidence that enhanced pathogen replication, transmission, and stress to the host are likely determinants (Taylor, 1983; Gilad et al., 2003; Lokmer and Wegner, 2015; Tout et al., 2015).

Laboratory- and field-based studies have identified clear temperature thresholds that facilitate pathogen transmission. For the pathogens *R. crassostreae, M. refringens* and OsHV-1, the highest levels of transmission occur at temperatures greater than 18 °C (Lewis et al., 1996), 17 °C (Audemard et al., 2001), and 13.4 °C (Petton et al., 2013) respectively. In the field, disease outbreaks by these pathogens occur at slightly elevated temperatures, exceeding 20 °C for ROD and marteiliosis (Berthe et al., 1998; Boettcher et al., 1999), and 16 °C for OsHV-1 (Renault et al., 2014), indicating that pathogen colonisation is only one aspect of disease causation and that conditions that favour growth and increased host susceptibility also drive outbreaks. Consistent with this, ROD disease onset is reduced from 7 weeks at the temperature permissible temperature of 18 °C to only 3 weeks when the temperature is increased to 25.9 °C, following transmission at 18 °C (Lewis et al., 1996). Regarding effects on the oyster host, warmer temperatures of 21 °C are sufficient to reduce the numbers of hemocytes in the *C. gigas* hemolymph, as well as reducing their phagocytic ability, as was demonstrated by oyster hemocytes challenged with *V. anguillarum* (Malham et al., 2009).

In contrast to the examples above, some pathogens have greater impacts under cooler temperatures. The viability of *M. sydneyi* spores is highest when temperature is reduced from 25 °C to 15 °C (Wesche et al., 1999), while *B. ostreae* shows improved survivability at 4 °C compared to temperatures at 15 °C and above (Arzul et al., 2009). Furthermore, outbreaks of winter mortality disease routinely occur in late winter or early spring (Roughley, 1926; Spiers et al., 2014).

5.2. Salinity

Salinity shifts have been implicated as key factors in outbreaks of dermo, MSX, ROD, QX, bonamiasis, vibriosis and summer mortality. Each oyster species has an optimal salinity concentration for growth, with 15–18 ppt (parts per thousand), 20–25 ppt, 20 ppt and 25–35 ppt being the optimal range for *C. virginica*, *C. gigas*, *O. edulis* and *S. glomerata* respectively (Nell and Holliday, 1988; Wallace, 2001; Fao, 2016a; c). Shifts from these optimal ranges can occur following rainfall events, periods of extended drought, tidal changes and from wind-driven flow (Geyer, 1997; Drexler and Ewel, 2001; Schmidt and Luther, 2002; Da Costa et al., 2016). Infections from dermo routinely occur at salinities above 9 ppt, with the greatest infections occurring above 15 ppt (Burreson and Ragone Calvo, 1996), which is within the optimal range of growth for *C. virginica* (Wallace, 2001), although once an oyster is infected, the infection can persist under salinity levels as low as 1–13 ppt (Andrews and Hewatt, 1957). Long periods of minimal rainfall, also lead to an increase in dermo disease intensity and prevalence, which is thought to be related to increased salinity levels (Soniat et al., 2012).

For *P. marinus* (> 15 ppt) and *H. nelsoni* (> 15 ppt), infections occur within the optimal range of growth for their host (15–18 ppt for *C. virginica*). MSX disease severity is increased when the salinity is greater than 15 ppt, which is also within the optimal salinity range for *C. virginica* (Haskin and Ford, 1982). The protozoan, *B. ostreae* and the spores of *M. sydneyi* prefer high salinity (Wesche et al., 1999; Arzul et al., 2009). *M. sydneyi* spores showing heightened viability with increasing salinity, with an optimum viability at 34 ppt (Wesche et al., 1999) corresponding to the optimal salinity range of 25–35 ppt for *S. glomerata*. *B. ostreae* shows greatest survival in salinities greater than 35 ppt (Arzul et al., 2009), which is beyond the optimal salinity concentration (20 ppt) for *O. edulis*.

Salinity levels can also impact bacterial diseases such as ROD and vibriosis. Transmission of ROD readily occurs at salinities greater than 18 ppt, the upper limit for *C. virginica*, and while infections do occur at lower salinities (10 ppt and 14 ppt) mortality rates are significantly decreased (Lewis et al., 1996). Conversely, mortality from *V. coralliilyticus* and *V. tubiashii* infection in *C. virginica* decreased from 100% to 70.7% respectively to 0% by reducing the salinity levels from 28 ppt to 9.6 ppt (Richards et al., 2015). Rates of summer mortality are also correlated with low salinity, with oyster mortalities the greatest during the low autumn-winter salinity period (Soletchnik et al., 2007).

With the exception of *B. ostreae*, the salinity concentrations that allow for infections by the protozoans are within the optimal range for their host. While bacterial infection and mortality caused by *R. crassostreae* (> 18 ppt), *V. coralliilyticus* (28 ppt) and *V. tubiashii* (28 ppt) all occur outside the hosts optimal salinity range (15–18 ppt) possibly indicating that bacteria require an external stressor to allow for disease progression to occur, while protozoan parasites do not.

5.3. Dissolved oxygen and pH

N. crassostreae induced mortalities are correlated with lower dissolved oxygen concentrations, possibly through an impact on the hosts ability to combat this pathogen (Engelsma et al., 2008). In addition, hypoxic environments have been shown to increase the acquisition and infection intensity of *P. marinus* infections in *C. virginica* (Breitburg et al., 2015; Keppel et al., 2015), while pH does not appear to play a role in *P. marinus* infection dynamics (Keppel et al., 2015). Decreased pH levels also significantly affect the formation and dissolution of the *C. virginica* shell, which can potentially increase oyster susceptibility to disease and predation (Waldbusser et al., 2011a, 2011b). The combination of decreased pH and a hypoxic environment reduces the ability of hemocytes to create reactive oxygen species (Boyd and Burnett, 1999), which would ultimately hamper their ability to combat microbial infections. Previous studies have shown that acidification of water (< pH 5.5) from acid sulphate soil runoff can reduce *S. glomerata* growth, degenerate oyster tissues and lead to higher mortality rates (Dove and Sammut, 2007a; b). In contrast, another study observed no correlation between pH and *M. sydneyi* infection of *S. glomerata* (Anderson et al., 1994), possibly indicating that pH is more influential on the *S. glomerata* oyster host, rather than influencing the protozoan parasite itself. In addition, *S. glomerata* acclimated to acidic water through the incorporation of CO_2 into the oyster rearing tanks were shown to have a reduced tolerance to shifting salinity levels and temperature (Parker et al., 2017).

5.4. Nutrients

The possible role of nutrients in summer mortality disease outbreaks was first considered in the 1960's, when outbreaks of summer mortality in *C. gigas* occurred in the Matsushima Bay, Japan, a region subject to heavy eutrophication (Mori, 1979). However, since this initial evidence, the role of nutrients in oyster disease and mortality events has rarely been directly studied. Concentrations of phosphate, nitrate, nitrite, silicate and ammonium were elevated during *C. gigas* summer mortality outbreaks in Ireland and Wales, while in subsequent laboratory experiments mortality of oysters from these environments was only induced following the additions of elevated nutrient concentrations (Malham et al., 2009). To our knowledge, this is the only study to examine the role of nutrients on oyster disease in depth. Although, a previous study has shown that growing oysters in nutrient enriched seawater led to mortality rates five times greater than those oysters in non-enriched seawater (Lipovsky and Chew, 1972). In a more general context, the role of nutrients, specifically from oyster feed, on oyster larval growth and survival has previously been reviewed (Marshall et al., 2010), with a general pattern of larvae diet strongly influencing larvae survival, as well as the need to supplement the larvae diet with protein as they progress through their life cycle (Marshall et al., 2010).

5.5. Translocation

While not an environmental factor, translocation is a common practice in the aquaculture industry and can unknowingly introduce pathogens to naïve areas. Examples of previous introductions of disease include marteiliosis and dermo (Alderman, 1979; Friedman and Perkins, 1994). Marteiliosis was spread from one affected area to other parts of France and then Spain, resulting in the introduction of *M. refringens* to these areas (Alderman, 1979). Dermo was historically located in the Chesapeake Bay, but persistent introductions of infected oysters to the north-eastern USA led to the establishment of dermo in these areas (Friedman and Perkins, 1994; Ford, 1996). Often though, translocation alone is not sufficient. Environmental conditions must be favourable to the pathogen to facilitate disease establishment and progression (Ford, 1996).

6. The relationship between the oyster microbiome and disease

Evidence for the importance of the microbiome has been building since the term “microbiome” was first coined in 1988 (Lisansky, 1988). Arguably, the bulk of the microbiome research has been focussed on humans, with specific compositions of the human gut microbiome correlated with a number of disorders/diseases (Turnbaugh et al., 2006; Abraham and Cho, 2009; Heijtz et al., 2011). In other organisms, the microbiome influences animal behaviour and their susceptibility to pathogens (Hosokawa et al., 2008; Koch and Schmid-Hempel, 2011), for example, the microbiome of *Drosophila melanogaster* (fruit fly) strongly drives the mating behaviour of this insect (Sharon et al., 2010). Using these examples, it is likely that the microbiome of oysters also plays a key role in oyster health, behaviour or through some contribution to the oyster disease process.

The role of the oyster microbiome in mortality outbreaks is an area of research yet to be fully explored. To date, previous research has shown that the microbiome can shift under a multitude of different stress treatments, such as translocation, starvation, temperature, infection and antibiotic stress (Green and Barnes, 2010; Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016a, 2016b). The microbiome also changes with different seasons (Pierce et al., 2016) and with translocation to laboratory conditions (Lokmer et al., 2016a). Additionally, while external abiotic factors can influence the microbiome, the within microbiome-interactions (between microbial organisms within a microbiome) can also play a role in bacterial community composition (Lokmer et al., 2016a) and destabilisation of this community can facilitate infection by *Vibrio* pathogens (Lokmer et al., 2016b) – this raises questions regarding the role of the oyster microbiome in disease resistance and susceptibility. Studies exploring the oyster microbiome during disease events are biased towards *C. gigas* and further towards summer mortality and the *Vibrio*-specific community.

The oyster microbiome is comprised of unique bacterial communities in each tissue, with the hemolymph bacterial community the most variable (King et al., 2012; Lokmer et al., 2016b). It has previously

been proposed that destabilisation of the hemolymph microbiome can allow *Vibrio* bacteria to infiltrate the solid tissues causing a systemic infection (Lokmer et al., 2016b). There is increasing evidence that the microbiome of an organism plays an essential role in maintaining homeostasis (Shin et al., 2011; Earley et al., 2015). For instance, in humans the microbiome maintains immune homeostasis through reduction of inflammation (Kelly et al., 2004), provides host microbial defence (Fukuda et al., 2011), assists in nutrient degradation and uptake (Turnbaugh et al., 2009) and microbiome imbalances have been linked to chronic diseases such as Crohn's disease (Frank et al., 2007). The role of the microbiome in disease dynamics is emerging as an important factor in the progression and severity of oyster diseases (Petton et al., 2015). Reduced mortality in antibiotic-treated specific-pathogen-free (SPF) oysters subsequently exposed to OsHV-1 suggests an important role for the oysters microbiome in disease dynamics (Petton et al., 2015), in particular, the *Vibrio* community in healthy *C. gigas* harbours pathogens that can induce mortality in oyster larvae (Wendling et al., 2014). Furthermore, the non-virulent *Vibrio* portion of the oyster microbiome progressively shifts towards a virulent population during the onset of summer mortality while the remaining non-virulent *Vibrio* population appears to aid in causing the disease (Lemire et al., 2015). When virulent *Vibrio* strains are injected into oysters, the oyster microbiome does not become dominated by *Vibrio,* in fact, organisms from the genus *Arcobacter* become dominant (Lokmer and Wegner, 2015). Similarly, by growing the *Vibrio*-injected oysters at higher temperatures (22 °C), the microbiome became more variable, with an increase in anaerobic bacteria, including members of the *Clostridia*, which were found to be a particularly large component of the microbial assemblage in dead oysters, possibly due to necrosis or anaerobic conditions (Lokmer and Wegner, 2015). From the few studies focussed on examining the *C. gigas* microbiome during a summer mortality disease outbreak, we can begin to make insights into how the native microbial community can facilitate disease progression. *C. gigas* cultivated at sites experiencing a summer mortality outbreak in Australia had a significantly different microbiome structure than specimens from sites unaffected by summer mortality (King et al., 2018) however, further research is required to determine the role of the whole microbiome in disease dynamics. There is evidence that shifts in the *Vibrio* community can increase the severity of disease, but it is unclear whether the whole microbial community, when stressed, provides a protective role against disease, or aids in disease progression (Thurber et al., 2009; Lemire et al., 2015; Tout et al., 2015).

To our knowledge, there has only been one study characterising the microbiome of *S. glomerata* during a disease event, with evidence that infection by *M. sydneyi* reduces the diversity of the oyster microbiome, with sequences with high homology to *Rickettsiale*-like prokaryotes highly elevated in infected oysters (Green and Barnes, 2010). Changes in the microbiome of *S. glomerata* in response to infection by *M. sydneyi* could further aid disease progression but further studies are required to examine whether mortality can be reduced in infected oysters with a more 'stable' microbiome.

The microbiome of *C. virginica* is understudied, particularly within the context of disease. To date, the culture-able bacterial community has been studied in regards to its oil degradation ability from the horizon oil spill in the Gulf of Mexico, with members of the *Pseudomonas* genus as the dominant oil-degrading isolate (Thomas et al., 2014), and the microbiome of *C. virginica* has been previously characterised using culture-independent techniques, in which the oyster gut microbiome (intestinal contents) was found to more diverse than the stomach microbiome, and the microbiome assemblage was influenced by spatial location (King et al., 2012; Chauhan et al., 2014). A recent spatiotemporal study of the *C. virginica* microbiome considered the influence of Dermo (Pierce et al., 2016). The *C. virginica* microbiome was shown to change over seasons, with the microbial community composition significantly influenced by water temperature, but the infection and severity of dermo disease was not found to be a significant determining factor of the microbiome (Pierce et al., 2016).

Similar to *S. glomerata* and *C. virginica,* studies of the *O. edulis* microbiome during disease events are lacking, indeed, studies characterising the healthy microbiome of *O. edulis* are also needed. To our knowledge, only one such study has examined the microbiome of *O. edulis,* with a focus on characterising the culture-able microbiome to examine shifts in the bacterial population over seasons, with isolates belonging to *Vibrio harveyi* dominant through the warmer months and *Vibrio splendidus* dominant during the colder months (Pujalte et al., 1999).

6.1. Oyster microbiome - future directions and challenges

Observational microbiome studies of *C. gigas* have begun to shed light on the dynamic interplay between the oyster microbiome, health, and disease. However, these studies are largely under-represented for *S. glomerata*, *C. virginica*, and *O. edulis*. It is becoming clear that applying stress to an oyster is sufficient to shift the oyster microbiome. This is seen with bacterial infection and temperature (Lokmer and Wegner, 2015), translocation (Lokmer et al., 2016b), starvation (Lokmer and Wegner, 2015), antibiotic stress (Lokmer et al., 2016a), exposure to a disease outbreak (King et al., 2018), and parasite infection (Green and Barnes, 2010). But it is not understood how the oyster microbiome responds before, during and after an environmental disease outbreak. Understanding this dynamic is crucial for determining the microbiome contribution to disease, and whether it can 'stabilise' following stress periods. However, carrying out environmental temporal studies are particularly challenging for a number of reasons: Firstly, in many cases the onset of disease can be very sudden and unpredictable. Secondly, holding/studying oysters in marine mesocosms (i.e. tanks or aquariums) significantly alters the oyster microbiome (Lokmer et al., 2016a) and will not be representative of an environmental outbreak. Thirdly, the oyster microbiome is highly heterogenous between replicate oysters (Lokmer et al., 2016a; King et al., 2018). Lastly, repeated hemolymph sampling of the same individual can cause local tissue infections resulting in an over-representation of bacteria assigned to the *Tenericutes* phylum (Lokmer et al., 2016a). To overcome these challenges, environmental temporal studies will need to have a high-resolution sampling regimen to capture the mortality event, likely coupled with a large number of biological replicates to overcome the heterogeneity in the oyster microbiome.

Breeding for disease resistance is a common aquaculture practice for the mitigation of oyster disease outbreaks (Dégremont, 2011; Dove et al., 2013b). Given the likely contribution of the oyster microbiome in oyster diseases (Lemire et al., 2015; Petton et al., 2015), there is a need to determine whether breeding for disease resistance also alters the oyster microbiome composition and whether this alteration is, at least in part, responsible for disease resistance. If indeed the microbiome does play a role in disease resistance, another question is whether disease resistance oysters bred in one aquatic environment translate to another with different environmental parameters and likely microbiota? In the first instance, identifying whether disease resistance oysters have unique microbiomes will provide some insights into its protective role and stability after a disease event. Most importantly, characterising disease resistant oyster microbiomes may identify probiotic targets for the use in disease management strategies. However, as each tissue (including the hemolymph) has their own unique microbiome (Lokmer et al., 2016b), studies aiming to identify microbes unique to disease resistant oysters might need to homogenise the oyster or use a multi-tissue approach.

Moving beyond observational microbiome studies to manipulative experiments is another key challenge. Observational studies can provide insights into which microbes are driving shifts in the microbiome and be correlated to factors such as disease resistance, but do not provide information on the functional genes playing a role in the interactome. Metagenomics has emerged as a potential but expensive

replacement for 16S rRNA microbiome sequencing (Handelsman, 2004). This technique provides both observational and functional data for microbiome analysis (Quince et al., 2017). However, as extracted DNA will contain a high ratio of eukaryotic to prokaryotic DNA, enrichment of prokaryotic DNA is required before sequencing (Thoendel et al., 2016).

Once the potential functional role of these microbes has been established, another key challenge is the cultivation and manipulation of specific members of the oyster microbiome. Cultivated organisms are required to characterise the interactions between these microbes (such as those correlated to disease resistance), the host, and pathogens (Bäumler and Sperandio, 2016), and to examine the probiotic effect of these microbes (Kapareiko et al., 2011). This may identify specific genetic elements that amplify or suppress oyster diseases, allowing for the development of monitoring programs to examine the abundance of these microbes/elements in commercial stocks and breeding programs.

7. Conclusions

Infectious diseases afflicting oysters have remained a constant barrier for the successful growth and sustainability of oyster aquaculture industries around the world. It is becoming increasingly apparent that the environment is an important factor driving the progression and severity of numerous oyster diseases and therefore, it is vital to consider how the environment can affect pathogen invasion and host physiology when studying oyster diseases. Oysters exist in an ever-changing environment and are constantly exposed to new challenges. In fact, the history of oyster cultivation is riddled with attempts to overcome new and existing oyster diseases (René Robert et al., 2013). While the bulk of previous research has been focused on the presence of aetiological agents and their link to mortality outbreaks, future studies should begin to question why these mortality outbreaks happen, what stimulates them, and how can these mortality outbreaks be lessened by manipulating the conditions in which oysters are grown in. Furthermore, how does the microbiome fit into the disease process? Previous research has shown that the oyster microbiome can shift under a multitude of conditions, some of these conditions, such as infection stress, are able to completely replace commensal members of the microbiome with a more virulent community (Lemire et al., 2015), and microbiome destabilisation can facilitate pathogen spill over into different oyster tissues (Lokmer et al., 2016b). This virulent state can then amplify the severity of oyster diseases. Disruption of the *C. gigas* microbiome during summer mortality outbreaks is emerging as an important factor determining the progression and severity of this disease. Yet, microbiome research in other oyster species, and their role in disease, is lacking. As an oyster is exposed to a dynamic environment, the microbes they are exposed to will change, both over seasons (Wendling et al., 2014) and with climate change. Will a changing environment completely change the oyster microbiome? Will it result in more microbiome disruptions, allowing diseases to take hold more frequently? Or perhaps the oyster microbiome is more resilient than previously thought? Here we have begun to tease apart the interconnectedness of the external environment and oyster diseases, yet it is still unclear whether the external environment acts directly on the oyster physiology and microbiome, allowing pathogens to take hold, or whether it only regulates pathogen proliferation and infection, which will cause disease regardless of the state of the oyster and its microbiome state. Answering these questions will provide vital insights into the complexity of oyster diseases and in turn, will guide management practices of oyster aquaculture to reduce the economic impact of these debilitating oyster diseases.

Acknowledgments

This work was supported by an Australian Research Council Linkage Project (LP160101785) to JRS, CJ and ML; 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 between the University of Technology Sydney and the New South Wales Department of Primary Industries.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2018.11.007.

References

Abraham, C., Cho, J.H., 2009. Inflammatory bowel disease. N. Engl. J. Med. 361, 2066–2078.
Adlard, R., Ernst, I., 1995. Extended range of the oyster pathogen, *Marteilia sydneyi*. Bull. Eur. Assoc. Fish Pathol. 15, 119–121.
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, 419–426.
Alderman, D., 1979. Epizootiology of *Marteilia refringens* in Europe. Mar. Fish. Rev. 41, 67–69.
Allam, B., Pales Espinosa, E., 2016. Bivalve immunity and response to infections: are we looking at the right place? Fish Shellfish Immunol. 53, 4–12.
Allam, B., Carden, W.E., Ward, J.E., Ralph, G., Winnicki, S., Pales Espinosa, E., 2013. Early host-pathogen interactions in marine bivalves: evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. J. Invertebr. Pathol. 113, 26–34.
Anderson, T.J., Wesche, S., Lester, R.J., 1994. Are outbreaks of *Marteilia sydneyi* in Sydney rock oysters, *Saccostrea commercialis*, triggered by a drop in environmental pH? Aust. J. Mar. Freshw. Res. 45, 1285–1287.
Andrews, J.D., Hewatt, W.G., 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake bay. Ecol. Monogr. 27, 2–25.
Arthur, R.F., Gurley, E.S., Salje, H., Bloomfield, L.S.P., Jones, J.H., 2017. Contact structure, mobility, environmental impact and behaviour: the importance of social forces to infectious disease dynamics and disease ecology. Phil. Trans. Roy. Soc. B. Biol. Sci. 372.
Arzul, I., Renault, T., Lipart, C., Davison, A.J., 2001. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J. Gen. Virol. 82, 865–870.
Arzul, I., Gagnaire, B., Bond, C., Chollet, B., Morga, B., Ferrand, S., Robert, M., Renault, T., 2009. Effects of temperature and salinity on the survival of *Bonamia ostreae*, a parasite infecting flat oysters *Ostrea edulis*. Dis. Aquat. Org. 85, 67–75.
Arzul, I., Langlade, A., Chollet, B., Robert, M., Ferrand, S., Omnes, E., Lerond, S., Couraleau, Y., Joly, J.P., François, C., Garcia, C., 2011. Can the protozoan parasite *Bonamia ostreae* infect larvae of flat oysters *Ostrea edulis*? Vet. Parasitol. 179, 69–76.
Arzul, I., Chollet, B., Boyer, S., Bonnet, D., Gaillard, J., Baldi, Y., Robert, M., Joly, J.Â.P., Garcia, C., Bouchoucha, M., 2014. Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*. Parasitology 141, 227–240.
Audemard, C., Barnaud, A., Collins, C.M., Le Roux, F., Sauriau, P.G., Coustau, C., Blachier, P., Berthe, F.C.J., 2001. Claire ponds as an experimental model for *Marteilia refringens* life-cycle studies: new perspectives. J. Exp. Mar. Biol. Ecol. 257, 87–108.
Audemard, C., Le Roux, F., Barnaud, A., Collins, C., Sautour, B., Sauriau, P.G., De Montaudouin, X., Coustau, C., Combes, C., Berthe, F., 2002. Needle in a haystack: involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*. Parasitology 124, 315–323.
Bachere, E.H., Mialhe, Dominique, Eric, 1991. Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. Dis. Aquat. Org. 11, 173–180.
Balouet, G., Poder, M., Cahour, A., 1983. Haemocytic parasitosis: morphology and pathology of lesions in the French flat oyster, Ostrea edulis L. Aquaculture 34, 1–14.
Bäumler, A.J., Sperandio, V., 2016. Interactions between the microbiota and pathogenic bacteria in the gut. Nature 535, 85–93.
Berthe, F.C.J., Pernas, M., Zerabib, M., Haffner, P., Thébault, A., Figueras, A.J., 1998. Experimental transmission of *Marteilia refringens* with special consideration of its life cycle. Dis. Aquat. Org. 34, 135–144.
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, 627–636.
Boardman, C.L., Maloy, A.P., Boettcher, K.J., 2008. Localization of the bacterial agent of juvenile oyster disease (*Roseovarius crassostreae*) within affected eastern oysters (*Crassostrea virginica*). J. Invertebr. Pathol. 97, 150–158.
Boettcher, K.J., Barber, B.J., Singer, J.T., 1999. Use of antibacterial agents to elucidate the etiology of juvenile oyster disease (JOD) in *Crassostrea virginica* and numerical dominance of an α- proteobacterium in JOD-affected animals. Appl. Environ. Microbiol. 65, 2534–2539.
Bougrier, S., Geairon, P., Deslous-Paoli, J.M., Bacher, C., Jonquières, G., 1995. Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). Aquaculture 134, 143–154.
Bower, S.M., 2006. Synopsis of infectious diseases and parasites of commercially exploited shellfish: Nocardiosis of Oysters. *Fisheries And Oceans Canada*.
Bower, S.M., 2011. Synopsis of infectious diseases and parasites of commercially exploited shellfish: Marteiliosis (Aber Disease) of Oysters. Department of Fisheries

and Oceans Canada.
Bower, S.M., Hervio, D., Meyer, G.R., 1997. Infectivity of *Mikrocytos mackini*, the causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas*, to various species of oysters. DAO (Dis. Aquat. Org.) 29, 111–116.
Boyd, J.N., Burnett, L.E., 1999. Reactive oxygen intermediate production by oyster hemocytes exposed to hypoxia. J. Exp. Biol. 202, 3135–3143.
Breitburg, D.L., Hondorp, D., Audemard, C., Carnegie, R.B., Burrell, R.B., Trice, M., Clark, V., 2015. Landscape-level variation in disease susceptibility related to shallow-water hypoxia. PLoS One 10 e0116223.
Bricelj, V.M., Ford, S.E., Borerro, F.J., Perkins, F.O., Rivara, G., Hillman, R.E., Elston, R.A., Chang, J., 1992. Unexplained mortalities of hatchery-reared, juvenile oysters, *Crassostrea virginica* (Gmelin). J. Shellfish Res. 11, 331–347.
Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., Polz, M.F., Le Roux, F., 2017. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. ISME J. 11.
Burge, C.A., Griffin, F.J., Friedman, C.S., 2006. Mortality and herpesvirus infections of the pacific oyster *Crassostrea gigas* in tomales bay, California, USA. Dis. Aquat. Org. 72, 31–43.
Burge, C.A., Judah, L.R., Conquest, L.L., Griffin, F.J., Cheney, D.P., Suhrbier, A., Vadopalas, B., Olin, P.G., Renault, T., Friedman, C.S., 2007. Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: the influence of oyster stock, planting time, pathogens, and environmental stressors. J. Shellfish Res. 26, 163–172.
Burreson, E.M., Ragone Calvo, L.M., 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. J. Shellfish Res. 15, 17–34.
Butt, D., Shaddick, K., Raftos, D., 2006. The effect of low salinity on phenoloxidase activity in the Sydney rock oyster, *Saccostrea glomerata*. Aquaculture 251, 159–166.
Camacho, A.F., Villalba, A., Beiras, R., Labarta, U., 1997. Absorption efficiency and condition of cultured mussels (*Mytilus Edulis* Galloprovincialis Linnaeus) of Galicia (NW Spain) infected by parasites *Marteilia refringens* grizel et al. and *Mytilicola intestinalis* steuer. J. Shellfish Res. 16, 77–82.
Canesi, L., Gallo, G., Gavioli, M., Pruzzo, C., 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Tech. 57, 469–476.
Carnegie, R.B., Burreson, E.M., 2011. Declining impact of an introduced pathogen:-*Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. Mar. Ecol. Prog. Ser. 432, 1–15.
Carnegie, R.B., Hill, K.M., Stokes, N.A., Burreson, E.M., 2014. The haplosporidian *Bonamia exitiosa* is present in Australia, but the identity of the parasite described as *Bonamia* (formerly Mikrocytos) *roughleyi* is uncertain. J. Invertebr. Pathol. 115, 33–40.
Chase, E., Young, S., Harwood, V.J., 2015. Sediment and vegetation as reservoirs of *Vibrio vulnificus* in the tampa bay estuary and Gulf of Mexico. Appl. Environ. Microbiol. 81, 2489.
Chauhan, A., Wafula, D., Lewis, D.E., Pathak, A., 2014. Metagenomic assessment of the Eastern oyster-associated microbiota. Genome Announc. 2.
Cheng, T.C., Rodrick, G.E., 1975. Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. Comp. Biochem. Physiol. B 52, 443–447.
Choi, K.S., Wilson, E.A., Lewis, D.H., Powell, E.N., Ray, S.M., 1989. The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. J. Shellfish Res. 8, 125–131.
Cook, T., Folli, M., Klinck, J., Ford, S., Miller, J., 1998. The relationship between increasing sea-surface temperature and the northward spread of *Perkinsus marinus* (Dermo) disease epizootics in oysters. Estuar. Coast Shelf Sci. 46, 587–597.
Da Costa, A.K.R., Pereira, L.C.C., Costa, S.F.S., Leite, N.R., De Flores-Montes, J.M., Da Costa, R.M., 2016. Spatiotemporal variation in salinity during drought years in an Amazonian estuary (Taperaçu). J. Coast Res. 1, 48–52.
De Decker, S., Saulnier, D., 2011. Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes. Fish Shellfish Immunol. 30, 691–699.
De Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., Haffner, P., Dégremont, L., Charrière, G.M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., Leroy, M.A., Pérignon, A., Régler, D., Morga, B., Alunno-Bruscia, M., Boudry, P., Le Roux, F., Destoumieux-Garzón, D., Gueguen, Y., Mitta, G., 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. Nat. Commun. 9, 4215.
Dégremont, L., 2011. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture 317, 94–98.
Dégremont, L., 2013. Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. Aquaculture 416–417, 129–134.
Dégremont, L., Tanguy, G., Delphine, T., Jean-François, P., 2013. Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles? Aquaculture 408–409, 51–57.
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, 509–518.
Dove, M.C., Sammut, J., 2007b. Impacts of estuarine acidification on survival and growth of Sydney rock oysters Saccostrea glomerata (Gould 1850). J. Shellfish Res. 26, 519–527.
Dove, M.C., Nell, J.A., Mcorrie, S., O'connor, W.A., 2013a. 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, 681–687.
Dove, M.C., Nell, J.A., O'connor, W.A., 2013b. 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*). Aquacult. Res. 44, 1791–1800.
Drexler, J.Z., Ewel, K.C., 2001. Effect of the 1997-1998 ENSO-related drought on hydrology and salinity in a Micronesian wetland complex. Estuaries 24, 347–356.
Earley, Z.M., Akhtar, S., Green, S.J., Naqib, A., Khan, O., Cannon, A.R., Hammer, A.M., Morris, N.L., Li, X., Eberhardt, J.M., Gamelli, R.L., Kennedy, R.H., Choudhry, M.A., 2015. Burn injury alters the intestinal microbiome and increases gut permeability and bacterial translocation. PLoS One 10.
Elston, R.A., Farley, C.A., Kent, M.L., 1986. Occurrence and significance of bonamiasis in European flat oysters *Ostrea edulis* in North America. Dis. Aquat. Org. 2, 49–54.
Elston, R.A., Hasegawa, H., Humphrey, K.L., Polyak, I.K., Hase, C.C., 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis. Aquat. Org. 82, 119–134.
Elston, R., Friedman, C., Gustafson, L., Meyer, G., Rogers, R., 2015. Denman island disease in Washington state, USA: distribution and prevalence in pacific and olympia oysters. Dis. Aquat. Org. 114, 147–154.
Encomio, V.G., Stickler, S.M., Allen Jr., S.K., Chu, F.L., 2005. Performance of "natural dermo-resistant" oyster stocks - survival, disease, growth, condition and energy reserves. J. Shellfish Res. 24, 143–155.
Engelsma, M.Y., Roozenburg, I., Joly, J.P., 2008. First isolation of *Nocardia crassostreae* from pacific oyster *Crassostrea gigas* in Europe. Dis. Aquat. Org. 80, 229–234.
Ewart, J.W., Ford, S.E., 1993. History and Impact of MSX and Dermo Diseases on Oyster Stocks in the Northeast Region. Northeastern Regional Aquaculture Center, University of Massachusett.
FAO, 2016a. Cultured Aquatic Species Information Programme. *Crassostrea gigas*. Cultured Aquatic Species Information Programme. FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en [online]. Text by Helm, M.M, (Accessed 4 February 2016).
FAO, 2016b. Cultured Aquatic Species Information Programme. *Crassostrea Virginica*. Cultured Aquatic Species Information Programme. FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en [online]. Text by Kennedy, V. S, (Accessed 4 February 2016).
FAO, 2016c. Cultured Aquatic Species Information Programme. *Ostrea edulis*. Cultured Aquatic Species Information Programme. FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Ostrea_edulis/en [online]. Text by Goulletquer, P, (Accessed 15 June 2016).
FAO, 2016d. Cultured Aquatic Species Information Programme. *Saccostrea Commercialis*. Cultured Aquatic Species Information Programme. FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Saccostrea_commercialis/en [online]. Text by John, A.N, (Accessed 16 May 2016).
FAO, 2016e. Hatchery Culture of Bivalves: a Practical Manual. *FAO Fisheries and Aquaculture Department*. http://www.fao.org/docrep/007/y5720e/y5720e00.htm [online]. Text by Helm, M.M. and Bourne, N, (Accessed 21 June 2016).
Farley, C.A., Wolf, P.H., Elston, 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, 581–593.
Ford, S.E., 1996. Range extension by the oyster parasite P*erkinsus marinus* into the northeastern United States: response to climate change? J. Shellfish Res. 15, 45–56.
Ford, S.E., Haskin, H.H., 1982. History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen in Delaware Bay, 1957-1980. J. Invertebr. Pathol. 40, 118–141.
Ford, S., Powell, E., Klinck, J., Hofmann, E., 1999. Modeling the MSX parasite in eastern oyster (*Crassostrea virginica*) populations. I. Model development, implementation, and verification. J. Shellfish Res. 18, 475–500.
Frank, D.N., St Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N., Pace, N.R., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc. Natl. Acad. Sci. U. S. A 104, 13780–13785.
Friedman, C.S., Hedrick, R.P., 1991. Pacific oyster nocardiosis: isolation of the bacterium and induction of laboratory infections. J. Invertebr. Pathol. 57, 109–120.
Friedman, C.S., Perkins, F.O., 1994. Range extension of *Bonamia ostreae* to Maine, U.S.A. J. Invertebr. Pathol. 64, 179–181.
Friedman, C.S., Beattie, J.H., Elston, R.A., Hedrick, R.P., 1991. Investigation of the relationship between the presence of a Gram-positive bacterial infection and summer mortality of the Pacific oyster, *Crassostrea gigas* Thunberg. Aquaculture 94, 1–15.
Friedman, C.S., Estes, R.M., Stokes, N.A., Burge, C.A., Hargove, J.S., Barber, B.J., Elston, R.A., Burreson, E.M., Reece, K.S., 2005. Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. Dis. Aquat. Org. 63, 33–41.
Fujiya, M., 1970. Oyster farming in Japan. Helgoländer Wissenschaftliche Meeresuntersuchungen 20, 464–479.
Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., Taylor, T.D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M., Ohno, H., 2011. *Bifidobacteria* can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547.
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, 187–196.
Gay, M., Renault, T., Pons, A.M., Le Roux, F., 2004. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: Taxonomy and host alterations. Dis. Aquat. Org. 62, 65–74.
Geyer, W.R., 1997. Influence of wind on dynamics and flushing of shallow estuaries. Estuar. Coast Shelf Sci. 44, 713–722.
Gilad, O., Yun, S., Adkison, M.A., Way, K., Willits, N.H., Bercovier, H., Hedrick, R.P., 2003. Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi.

J. Gen. Virol. 84, 2661–2668.
Go, J., Deutscher, A., Spiers, Z., Dahle, K., Kirkland, P., Jenkins, C., 2017. An investigation into mass mortalities of unknown aetiology in Pacific oysters, *Crassostrea gigas,* in Port Stephens, New South Wales, Australia. Dis. Aquat. Org. 125, 227–242.
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, 613–622.
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, 47–53.
Hada, H.S., West, P.A., Lee, J.V., Stemmler, J., Colwell, R.R., 1984. *Vibrio tubiashii* sp. nov., a Pathogen of Bivalve Mollusks. Int. J. Syst. Evol. Microbiol. 34, 1–4.
Handelsman, J., 2004. Metagenomics: Application of Genomics to Uncultured Microorganisms. Microbiol. Mol. Biol. Rev. 68, 669–685.
Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., Samuel, M.D., 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296, 2158–2162.
Haskin, H.H., Ford, S.E., 1982. *Haplosporidium nelsoni* (MSX) on delaware bay seed oyster beds: A host-parasite relationship along a salinity gradient. J. Invertebr. Pathol. 40, 388–405.
Haskin, H.H., Stauber, L.A., Mackin, J.A., 1966. *Minchinia nelsoni* n. sp. (*haplosporida, haplosporidiidae*): Causative agent of the delaware bay oyster epizoötic. Science 153, 1414–1416.
Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., Pettersson, S., 2011. Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. U. S. A 108, 3047–3052.
Hervio, D., Bower, S.M., Meyer, G.R., 1996. Detection, isolation, and experimental transmission of *Mikrocytos mackini*, a microcell parasite of pacific oysters *Crassostrea gigas* (Thunberg). J. Invertebr. Pathol. 67, 72–79.
Hine, P., Wesney, B., Hay, B., 1992. Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. Dis. Aquat. Org. 12, 135–142.
Hosokawa, T., Kikuchi, Y., Shimada, M., Fukatsu, T., 2008. Symbiont acquisition alters behaviour of stinkbug nymphs. Biol. Lett. 4, 45–48.
Itoh, N., Takahashi, K.G., 2008. Distribution of multiple peptidoglycan recognition proteins in the tissues of Pacific oyster, *Crassostrea gigas*. Comp. Biochem. Physiol B Comp. Biochem. Mol. Biol. 150, 409–417.
Jeffries, V.E., 1982. Three Vibrio strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. Aquaculture 29, 201–226.
Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'connor, W., Kirkland, P.D., Frances, J., 2013. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis. Aquat. Org. 105, 109–126.
Kapareiko, D., Lim, H.J., Schott, E.J., Hanif, A., Wikfors, G.H., 2011. Isolation and Evaluation of New Probiotic Bacteria for use in Shellfish Hatcheries: II. Effects of a *Vibrio* sp. Probiotic Candidate Upon Survival of Oyster Larvae (*Crassostrea virginica*) in Pilot-Scale Trials. J. Shellfish Res. 30, 617–625.
Keeling, S.E., Brosnahan, C.L., Williams, R., Gias, E., Hannah, M., Bueno, R., Mcdonald, W.L., Johnston, C., 2014. New Zealand juvenile oyster mortality associated with ostreid herpesvirus 1-an opportunistic longitudinal study. Dis. Aquat. Org. 109, 231–239.
Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., Conway, S., 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nat. Immunol. 5, 104–112.
Keppel, A.G., Breitburg, D.L., Wikfors, G.H., Burrell, R.B., Clark, V.M., 2015. Effects of co-varying diel-cycling hypoxia and pH on disease susceptibility in the eastern oyster *Crassostrea virginica*. Mar. Ecol. Prog. Ser. 538, 169–183.
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, e51475.
King, W.L., Jenkins, C., Go, J., Siboni, N., Seymour, J.R., Labbate, M., 2018. Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. Microb. Ecol. https://doi.org/10.1007/s00248-018-1226-9. In press.
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, 767–784.
Koch, R., 1884. Die Ätiologie der Tuberkulose. Mittheilungen aus dem Kaiserlichen Gesundheitsamte 428–445.
Koch, H., Schmid-Hempel, P., 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc. Natl. Acad. Sci. 108, 19288–19292.
Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, J.-L., 2006a. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microb. Infect. 8, 2715–2724.
Labreuche, Y., Soudant, P., Goncalves, M., Lambert, C., Nicolas, J.L., 2006b. Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and cellular immune responses of the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 30, 367–379.
Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M., Poulet, S.A., 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. Org. 46, 139–145.
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, 1523–1531.
Lester, R.J.G., 1986. Field and Laboratory Observations on the Oyster Parasite *Marteilia Sydneyi*. University of Queensland Press Parastie Lives: Papers on Parasites, Their Hosts and Their Associations, pp. 33–40.
Leung, T.L.F., Bates, A.E., 2013. More rapid and severe disease outbreaks for aquaculture at the tropics: implications for food security. J. Appl. Ecol. 50, 215–222.
Lewis, E.J., Farley, C.A., Small, E.B., Baya, A.M., 1996. A synopsis of juvenile oyster disease (JOD) experimental studies in *Crassostrea virginica*. Aquat. Living Resour. 9, 169–178.
Lipovsky, V.P., Chew, K.K., 1972. Mortality of Pacific oysters (*Crassostrea gigas*): the influence of temperature and enriched seawater on oyster survival. Proc. Natl. Shellfish. Assoc. 72–82.
Lisansky, S.G., 1988. In: Burge, M.N. (Ed.), Fungi in Biological Control Systems. Manchester University Press.
Löffler, F., 1884. Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe.
Lokmer, A., Wegner, K.M., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J. 9, 670–682.
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.
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, 970–987.
López-Sanmartín, M., Batista, F.M., Del Carmen Marín, M., Garrido, I., Quintero, D., Grade, A., Ruano, F., De La Herrán, R., Navas, J.I., 2015. Detection of *Marteilia refringens* infecting the European flat oyster *Ostrea edulis* and the dwarf oyster *Ostrea stentina* in southern Portugal and Spain. J. Invertebr. Pathol. 130, 52–55.
Lopez-Sanmartin, M., Lopez-Fernandez, J.R., Cunha, M.E., De La Herran, R., Navas, J.I., 2016. Ostreid herpesvirus in wild oysters from the Huelva coast (SW Spain). Dis. Aquat. Org. 120, 231–240.
Luna-González, A., Maeda-Martínez, A.N., Sainz, J.C., Ascencio-Valle, F., 2002. Comparative susceptibility of veliger larvae of four bivalve mollusks to a *Vibrio alginolyticus* strain. Dis. Aquat. Org. 49, 221–226.
Lynch, S.A., Flannery, G., Hugh-Jones, T., Hugh-Jones, D., Culloty, S.C., 2014. Thirty-year history of Irish (Rossmore) *Ostrea edulis* selectively bred for disease resistance to *Bonamia ostreae*. Dis. Aquat. Org. 110, 113–121.
Mackin, J.G., 1959. Mortalities in oysters. Proc. Natl. Shellfish. Assoc. 50, 21–40.
Mackin, J.G., Owen, H.M., Collier, A., 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). Science 111, 328–329.
Malham, S.K., Cotter, E., O'keeffe, S., Lynch, S., Culloty, S.C., King, J.W., Latchford, J.W., Beaumont, A.R., 2009. Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of temperature and nutrients on health and survival. Aquaculture 287, 128–138.
Marshall, R., Mckinley, S., Pearce, C.M., 2010. Effects of nutrition on larval growth and survival in bivalves. Rev. Aquacult. 2, 33–55.
Martenot, C., Oden, E., Travaille, E., Malas, J.P., Houssin, M., 2011. Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. Virus Res. 160, 25–31.
Martinez-Urtaza, J., Bowers, J.C., Trinanes, J., Depaola, A., 2010. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus illnesses*. Food Res. Int. 43, 1780–1790.
Mccallum, H., Harvell, D., Dobson, A., 2003. Rates of spread of marine pathogens. Ecol. Lett. 6, 1062–1067.
Montes, J., Anadón, R., Azevedo, C., 1994. A Possible Life Cycle for *Bonamia ostreae* on the Basis of Electron Microscopy Studies. J. Invertebr. Pathol. 63, 1–6.
Morga, B., Arzul, I., Chollet, B., Renault, T., 2009. Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*. Fish Shellfish Immunol. 26, 836–842.
Morga, B., Faury, N., Guesdon, S., Chollet, B., Renault, T., 2017. Haemocytes from *Crassostrea gigas* and OsHV-1: A promising in vitro system to study host/virus interactions. J. Invertebr. Pathol. 150, 45–53.
Mori, K., 1979. Effects of artificial eutrophication on the metabolism of the Japanese oyster *Crassostrea gigas*. Mar. Biol. 53, 361–369.
Mortensen, S., Strand, A., Bodvin, T., Alfjorden, A., Skar, C.K., Jelmert, A., Aspán, A., Sælemyr, L., Naustvoll, L.J., Albretsen, J., 2016. Summer mortalities and detection of ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. Dis. Aquat. Org. 117, 171–176.
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, 39–44.
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*. Aquacult. Res. 37, 693–700.
Nell, J.A., Smith, R.I., Mcphee, C.C., 2000. The Sydney rock oyster *Saccostrea glomerata* (Gould 1850) breeding programme: Progress and goals. Aquacult. Res. 31, 45–49.
Olafsen, J.A., Mikkelsen, H.V., Glaever, H.M., Hansen, G.H., 1993. Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. Appl. Environ. Microbiol. 59, 1848–1854.
O'connor, W., Dove, M., Finn, B., 2008. Sydney Rock Oysters: Overcoming Constraints to Commercial Scale Hatchery and Nursery Production. NSW Department of Primary Industries.
Pales Espinosa, E., Corre, E., Allam, B., 2014. Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus*. Int. J. Parasitol. 44, 305–317.
Parker, L.M., Scanes, E., O'connor, W.A., Coleman, R.A., Byrne, M., Pörtner, H.O., Ross, P.M., 2017. Ocean acidification narrows the acute thermal and salinity tolerance of

the Sydney rock oyster *Saccostrea glomerata*. Mar. Pollut. Bull. 122, 263–271.
Pawiro, S., 2010. Safe Management of Shellfish and Harvest Waters. World Health Organisation (WHO).
Perkins, F.O., 1996. The Structure of *Perkinsus Marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with Comments on Taxonomy and Phylogeny of *Perkinsus* Spp. *VIMS Articles* 506.
Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pépin, J.F., Keck, N., 2012. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquacult. Environ. Interact. 2, 215–237.
Pernet, F., Lupo, C., Bacher, C., Whittington, R.J., 2016. Infectious diseases in oyster aquaculture require a new integrated approach. Phil. Trans. Roy. Soc. B. Biol. Sci. 371.
Petton, B., Pernet, F., Robert, R., Boudry, P., 2013. Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. Aquacult. Environ. Interact. 3, 257–273.
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.
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, 97–113.
Pujalte, M.J., Ortigosa, M., Macian, M.C., Garay, E., 1999. Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int. Microbiol. 2, 259–266.
Quayle, D.B., 1961. Denman Island Disease and Mortality, 1960. *Fisheries Research Board of Canada,* Manuscript Report 713.
Quayle, D.B., 1988. Pacific oyster culture in British Columbia. Can. Bull. Fish. Aquat. Sci. 218.
Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., Segata, N., 2017. Shotgun metagenomics, from sampling to analysis. Nat. Biotechnol. 35, 833.
Ragone Calvo, L.M., Calvo, G.W., Burreson, E.M., 2003. Dual disease resistance in a selectively bred eastern oyster, *Crassostrea virginica*, strain tested in Chesapeake Bay. Aquaculture 220, 69–87.
Ralph, A.E., Paul, F., Dan, C., 1999. Extrapallial abscesses associated with chronic bacterial infections in the intensively cultured juvenile Pacific oyster *Crassostrea gigas*. Dis. Aquat. Org. 37, 115–120.
Renault, T., Cochennec, N., Le Deuff, R.-M., Chollet, B., 1994. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. Bull. Eur. Assoc. Fish Pathol. 14, 64–66.
Renault, T., Lipart, C., Arzul, I., 2001. A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. J. Fish. Dis. 24, 369–376.
Renault, T., Bouquet, A.L., Maurice, J.-T., Lupo, C., Blachier, P., 2014. Ostreid Herpesvirus 1 Infection among Pacific Oyster (*Crassostrea gigas*) Spat: Relevance of Water Temperature to Virus Replication and Circulation Prior to the Onset of Mortality. Appl. Environ. Microbiol. 80, 5419–5426.
René Robert, J.L.S., Pérez-Parallé, Luz, Ponis, Emanuele, Kamermans, Pauline, O'mahoney, M., 2013. A glimpse on the mollusc industry in Europe. Aquacult. Eur. 38, 5–11.
Richards, G.P., Watson, M.A., Needleman, D.S., Church, K.M., Hase, C.C., 2015. Mortalities of Eastern and Pacific oyster Larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. Appl. Environ. Microbiol. 81, 292–297.
Riisgård, H., 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Mar. Ecol. Prog. Ser. (Oldendorf) 45, 217–223.
Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. Aquaculture 172, 125–145.
Roubal, F.R., Masel, J., Lester, R.J.G., 1989. Studies on *marteilia sydneyi*, agent of QX disease in the sydney rock oyster, *Saccostrea commercialis* with implications for its life cycle. Mar. Freshw. Res. 40, 155–167.
Roughley, T.C., 1926. An investigation of the cause of an oyster mortality on the Georges River, New South Wales, 1924-25. Proc. Linn. Soc. N. S. W. 51, 446–491.
Samain, J.F., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J.L., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., Boudry, P., 2007. Genetically based resistance to summer mortality in the Pacific oyster (Crassostrea gigas) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture 268, 227–243.
Saulnier, D., De Decker, S., Haffner, P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. J. Microbiol. Methods 77, 191–197.
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, 787–798.
Schmidt, N., Luther, M.E., 2002. ENSO impacts on salinity in Tampa Bay, Florida. Estuaries 25, 976–984.
Schmitt, P., Duperthuy, M., Montagnani, C., Bachère, E., Destoumieux-Garzón, D., 2012a. Immune responses in the Pacific oyster Crassostrea gigas: An overview with focus on summer mortalities. In: Oysters: Physiology, Ecological Distribution and Mortality. Nova Science Publishers, Inc.), pp. 227–273.
Schmitt, P., Lorgeril, J.D., Gueguen, Y., Destoumieux-Garzón, D., Bachère, E., 2012b. Expression, tissue localization and synergy of antimicrobial peptides and proteins in the immune response of the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 37, 363–370.
Schmitt, P., Rosa, R.D., Duperthuy, M., De Lorgeril, J., Bachere, E., Destoumieux-Garzon, D., 2012c. The Antimicrobial Defense of the Pacific Oyster, *Crassostrea gigas*. How Diversity may Compensate for Scarcity in the Regulation of Resident/Pathogenic Microflora. Front. Microbiol. 3, 160.
Schott, E.J., Pecher, W.T., Okafor, F., Vasta, G.R., 2003. The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. Exp. Parasitol. 105, 232–240.
Schrobback, P., Pascoe, S., Coglan, L., 2015. History, status and future of Australia's native Sydney rock oyster industry. Aquat. Living Resour. 27, 153–165.
Segarra, A., Pepin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res. 153, 92–99.
Segarra, A., Mauduit, F., Faury, N., Trancart, S., Dégremont, L., Tourbiez, D., Haffner, P., Barbosa-Solomieu, V., Pépin, J.-F., Travers, M.-A., Renault, T., 2014. Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. BMC Genomics 15, 1–13.
Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I., Rosenberg, E., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 107, 20051–20056.
Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., Lee, W.J., 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334, 670–674.
Smith, I.R., Nell, J.A., Adlard, R., 2000. The effect of growing level and growing method on winter mortality, *Mikrocytos roughleyi*, in diploid and triploid Sydney rock oysters, Saccostrea glomerata. Aquaculture 185, 197–205.
Soletchnik, P., Lambert, C., Costil, K., 2005. Summer mortality of *Crassostrea gigas* (Thunberg) in relation to environmental rearing conditions. J. Shellfish Res. 24, 197–207.
Soletchnik, P., Ropert, M., Mazurié, J., Gildas Fleury, P., Le Coz, F., 2007. Relationships between oyster mortality patterns and environmental data from monitoring databases along the coasts of France. Aquaculture 271, 384–400.
Soniat, T.M., Klinck, J.M., Powell, E.N., Hofmann, E.E., 2012. Understanding the Success and Failure of Oyster Populations: Periodicities of *Perkinsus marinus*, and Oyster Recruitment, Mortality, and Size. J. Shellfish Res. 31, 635–646.
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. Org. 110, 151–164.
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, 111–118.
Sunila, I., Karolus, J., Lang, E.P., Mroczka, M.E., Volk, J., 2000. Transmission of the haplosporidian parasite MSX *Haplosporidium nelsoni* to the eastern oyster *Crassostrea virginica* in an upweller system. Dis. Aquat. Org. 42, 153–155.
Takahashi, K.G., Nakamura, A., Mori, K., 2000. Inhibitory effects of ovoglobulins on bacillary necrosis in larvae of the Pacific oyster, *Crassostrea gigas*. J. Invertebr. Pathol. 75, 212–217.
Taylor, D.L., 1983. The Black Band Disease of Atlantic Reef Corals. II. Isolation, Cultivation, and Growth of *Phormidium corallyticum*. Mar. Ecol. 4, 321–328.
Thoendel, M., Jeraldo, P.R., Greenwood-Quaintance, K.E., Yao, J.Z., Chia, N., Hanssen, A.D., Abdel, M.P., Patel, R., 2016. Comparison of microbial DNA enrichment tools for metagenomic whole genome sequencing. J. Microbiol. Methods 127, 141–145.
Thomas, J.C., Wafula, D., Chauhan, A., Green, S.J., Gragg, R., Jagoe, C., 2014. A survey of deepwater horizon (DWH) oil-degrading bacteria from the Eastern oyster biome and its surrounding environment. Front. Microbiol. 5.
Thurber, R.V., Willner-Hall, D., Rodriguez-Mueller, B., Desnues, C., Edwards, R.A., Angly, F., Dinsdale, E., Kelly, L., Rohwer, F., 2009. Metagenomic analysis of stressed coral holobionts. Environ. Microbiol. 11, 2148–2163.
Tout, J., Siboni, N., Messer, L.F., Garren, M., Stocker, R., Webster, N.S., Ralph, P.J., Seymour, J.R., 2015. Increased seawater temperature increases the abundance and alters the structure of natural *Vibrio* populations associated with the coral *Pocillopora damicornis*. Front. Microbiol. 6.
Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.
Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., Gordon, J.I., 2009. A core gut microbiome in obese and lean twins. Nature 457, 480–484.
Vezzulli, L., Pruzzo, C., Huq, A., Colwell, R.R., 2010. Environmental reservoirs of *Vibrio cholerae* and their role in cholera. Environ. Microbiol. Rep. 2, 27–33.
Vezzulli, L., Grande, C., Reid, P.C., Hélaouët, P., Edwards, M., Höfle, M.G., Brettar, I., Colwell, R.R., Pruzzo, C., 2016. Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. Proc. Natl. Acad. Sci. 113, E5062–E5071.
Virvilis, C., Angelidis, P., 2006. Presence of the parasite *Marteilia* sp. in the flat oyster (*Ostrea edulis* L) in Greece. Aquaculture 259, 1–5.
Waechter, M., Le Roux, F., Nicolas, J.L., Marissal, E., Berthe, F., 2002. Characterisation of *Crassostrea gigas* spat pathogenic bacteria. Comptes Rendus Biol. 325, 231–238.
Waldbusser, G.G., Steenson, R.A., Green, M.A., 2011a. Oyster shell dissolution rates in estuarine waters: Effects of ph and shell legacy. J. Shellfish Res. 30, 659–669.
Waldbusser, G.G., Voigt, E.P., Bergschneider, H., Green, M.A., Newell, R.I.E., 2011b. Biocalcification in the Eastern Oyster (*Crassostrea virginica*) in Relation to Long-term Trends in Chesapeake Bay pH. Estuar. Coast 34, 221–231.
Wallace, R.K., 2001. Cultivating the Eastern Oyster, *Crassostrea Virginica*. *USDA Southern Region Aquaculture Center (SRAC)* Publication No. 432.
Watermann, B.T., Herlyn, M., Daehne, B., Bergmann, S., Meemken, M., Kolodzey, H., 2008. Pathology and mass mortality of Pacific oysters, *Crassostrea gigas* (Thunberg),

in 2005 at the East Frisian coast, Germany. J. Fish. Dis. 31, 621–630.
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.
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, e94256.
Wesche, S.J., Adlard, R.D., Lester, R.J.G., 1999. Survival of spores of the oyster pathogen *Marteilia sydneyi* (Protozoa, Paramyxea) as assessed using fluorogenic dyes. Dis. Aquat. Org. 36, 221–226.
Wilson, M.E., 1995. Infectious diseases: an ecological perspective. BMJ 311, 1681–1684.

Microbial Ecology
https://doi.org/10.1007/s00248-018-1226-9

INVERTEBRATE MICROBIOLOGY

Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event

William L. King[1,2] · Cheryl Jenkins[3] · Jeffrey Go[3] · Nachshon Siboni[2] · Justin R. Seymour[2] · Maurizio Labbate[1]

Received: 5 November 2017 / Accepted: 2 July 2018

Abstract

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

Keywords *Crassostrea gigas* · Mortality · Oyster · Microbiome · Summer mortality

Introduction

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

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

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

It is notable, however, that in many instances of summer mortality, no clear aetiological agent has been identified [7,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1226-9) contains supplementary material, which is available to authorized users.

✉ Maurizio Labbate
maurizio.labbate@uts.edu.au

[1] The School of Life Sciences, University of Technology Sydney, PO Box 123, Broadway, Sydney, NSW 2007, Australia

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

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

Published online: 10 July 2018

17]. For these summer mortality events (and in fact for many other oyster diseases), a number of different environmental and physiological factors, including temperature, nutrient concentrations, chlorophyll *a* levels, turbidity, salinity, oyster growth rate and reproductive effort, have been implicated as triggers for mortality events [4, 8, 18–21]. However, in most cases, no single clear determinative factor(s) has been found. There is also evidence that the severity of summer mortality events is influenced by the host's genetic background, and this is being exploited for disease management by breeding resistant genetic lines [22–25].

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

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

In the summer of 2013 to 2014, a sudden mortality event occurred in the Port Stephens estuary, New South Wales, Australia. The New South Wales Department of Primary Industries (NSW DPI) obtained oysters as part of a structured survey to identify potential aetiological agents involved in this mortality event [17]. All oysters were found to be negative for the presence of OsHV-1 and OsHV-1 μvar and other known oyster pathogens [17]. Bacterial cultivation work identified numerous *Vibrio* species (*V. crassostreae*, *V. splendidus*, *V. harveyi* and *V. alginolyticus*) as being dominant in different sites, although no single clear dominant bacterium was consistently seen across the estuary [17]. Environmental data collected at the time indicated that sudden decreases in salinity due to rainfall and high temperature (> 20 °C) were likely contributors to the mortality event [17]. As no clear aetiological agent was found during the structured survey and to further explore this mortality event, we aimed to compare the microbiome of *C. gigas* oysters from disease-affected and disease-unaffected sites and to explore whether the oyster microbiome was influenced by spatial variation.

Methods

Oyster Study Sites and Cultivation

Port Stephens is a tide-dominated estuary [32], located on the east coast of New South Wales (NSW), Australia (32° 41′ 53.9″S 152° 01′ 26.3″E; Fig. 1). During a major mortality event that occurred in the austral summer of 2014 (January 6–13), *C. gigas* oysters were collected from leases where mortality was recorded. These leases were spread over seven localities within the Port Stephens estuary, of which five (Swan Bay, Karuah, Carrington, North Arm Cove and Tea Gardens) were situated in the northern part of the estuary and two (Cromartys Bay and Tilligerry Creek) in the southern part (Fig. 1). Amongst these seven localities, there were 12 sampling sites. Of these, 10 sites were cultivated for commercial purposes and affected by the mortality outbreak (will be referred to as disease-affected), whereas two sites (one in Cromartys Bay and one in Karuah) were disease-unaffected wild oysters (disease-unaffected will be referred to as unaffected). The southern part of the Port Stephens estuary contains the bulk of the urban population [33], with only Tea Gardens in the far north eastern part of the estuary with a comparable population size [33]; the southern sites also have a strong agricultural and mining industrial presence, particularly near Tilligerry Creek [34].

Site A in Cromartys Bay contained two stocks that were either affected or unaffected by the mortality event (Fig. 1). Site F in Tilligerry creek had two stocks with different final mortalities (10 and 99%). Due to the low mortality for this stock (10%) and the significantly different microbial assemblage (one-way ANOSIM using five samples per stock: $p = 0.0067$; $R = 0.61$ with 9999 permutations) to the 99% mortality stock, it was treated as an unaffected stock. The oyster cultivation conditions at each site (where known) are summarised in Table 1. Diploid oysters were cultivated at all sites, with the exception of one site in Tilligerry creek, where triploid oysters were cultivated. Oysters were predominately grown in trays except for those in North Arm Cove and Karuah (disease-affected site), which were grown in long-line baskets. Cultivated oysters were sourced from a Tasmanian hatchery, a Port Stephens-based hatchery, or wild-caught oyster seed or were wild non-cultivated oysters.

Oyster Sample Processing and DNA Extraction

Juvenile oysters were collected from each site and transported back to the laboratory in iced containers as previously described [17], where they were stored at − 80 °C prior to analysis. Five samples from each sampling

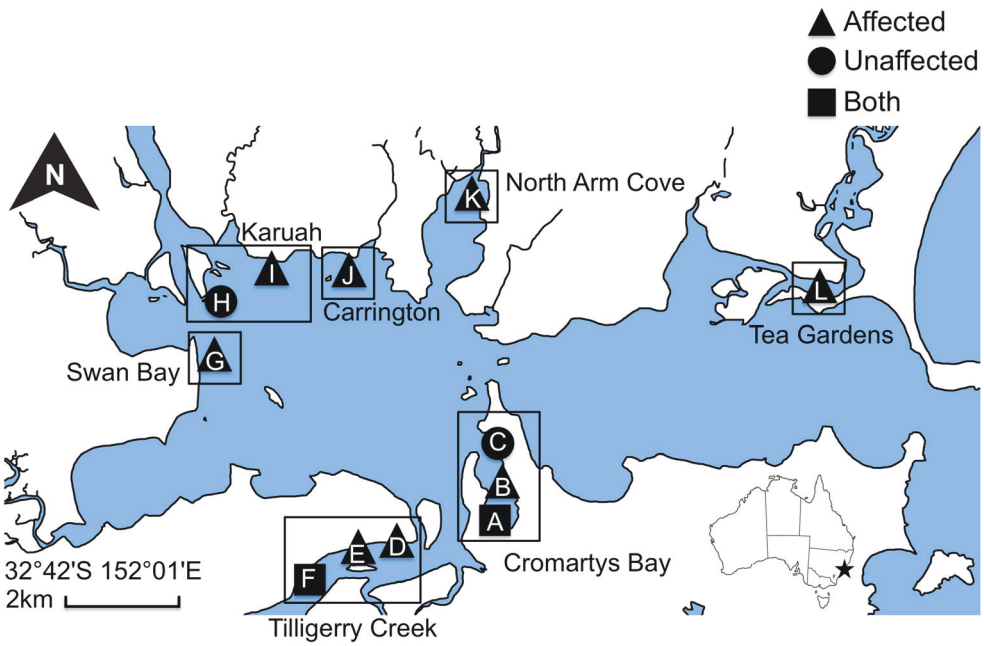


Fig. 1 Sampling locations and sites across the Port Stephens estuary. Areas with more than one sampling site are designated as localities and are contained within boxes. Sites are numbered A through to L, which corresponds to the site numbers in Table 1

site were thawed and scrubbed with a hard bristled brush under running water to remove any remaining mud and debris on the outer shell. Samples were then shucked using sterile shucking knives and immediately placed into sterile petri dishes. Approximately 25 mg of adductor muscle tissue was dissected and removed using sterile scalpel blades. Haemolymph collected via aspiration of the adductor muscle sinuses is frequently used to examine the bacterial population circulating within oysters [27, 28, 35, 36]; however, the use of frozen oysters in this study precluded aspiration of the haemolymph via syringe. Therefore, in line with the approaches used in several previous studies [27, 28, 35], aseptically dissected adductor muscle tissue containing haemolymph sinuses [37] was used to capture the fluid contained within.

DNA was extracted from the dissected adductor muscle using the Qiagen DNeasy Blood and Tissue Kit (catalogue 69506), as per the manufacturer's instructions. Extracted DNA was then amplified using PCR targeting the ribosomal 16S rRNA V1–V3 region using the 27F (5′-AGAG TTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′) primer pair [38, 39]. The PCR cycling conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. Amplicons were sequenced using the Illumina MiSeq platform (version 3; 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 (SRP139423) under Bioproject number PRJNA449563.

Data Analysis

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

Statistical Analyses

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

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

Results

Sequence Read Depth and Rarification

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

Replicates of *C. gigas* Microbiomes Show Large Within-Site Heterogeneity

We observed a high level of within-site variation in the composition of the *C. gigas* microbiome (Fig. 2). For instance, unaffected oyster microbiomes from site A (Table 1) exhibited low similarity (defined by a low median) between replicates (median ± standard deviation 0.13 ± 0.10), whilst disease-affected replicates in Tilligerry creek (site D; 98% mortality) had the lowest variability (defined by a low standard deviation) between individuals (0.27 ± 0.05). At Cromartys Bay site A, disease-affected oyster microbiomes had significantly less inter-oyster variability than the unaffected oysters (Kruskal-Wallis ANOVA, $p = 0.005$; 0.33 ± 0.09 and 0.13 ± 0.10, respectively; Supplementary Table 1); this was also observed at site F in Tilligerry Creek ($p = 0.007$; disease-affected 0.36 ± 0.097 and unaffected 0.21 ± 0.075). There was no significant difference when comparing variation between those oyster microbiomes in the north versus the south ($p = 0.29$). However, disease-affected microbiomes in the north had significantly less similarity between samples than those in the south ($p = 0.009$; 0.28 ± 0.089 and 0.34 ± 0.149, respectively). Differences in the local environment could potentially explain this difference in similarity, as the southern part of the estuary is largely urbanised and has a strong agricultural and mining presence [33].

C. gigas Microbiomes From the Port Stephens Mortality Event Separate Both Spatially and in Accordance With Disease State

We sought to examine whether the microbial assemblage of oyster microbiomes were influenced by disease-state, spatial location and stock source. The *C. gigas* microbiome composition significantly differed according to spatial location and disease state (Fig. 3). Oyster samples taken from sites experiencing no mortalities had a significantly different microbiome composition than samples from disease-affected sites in the north ($p = 0.0002$; $R = 0.26$) and south ($p = 0.0001$; $R = 0.21$). In addition, oysters collected from sites exhibiting high mortalities within the southern region of the estuary

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

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

[a] Information not supplied by the oyster farmer

[b] Wild oysters were not grown on a commercial lease

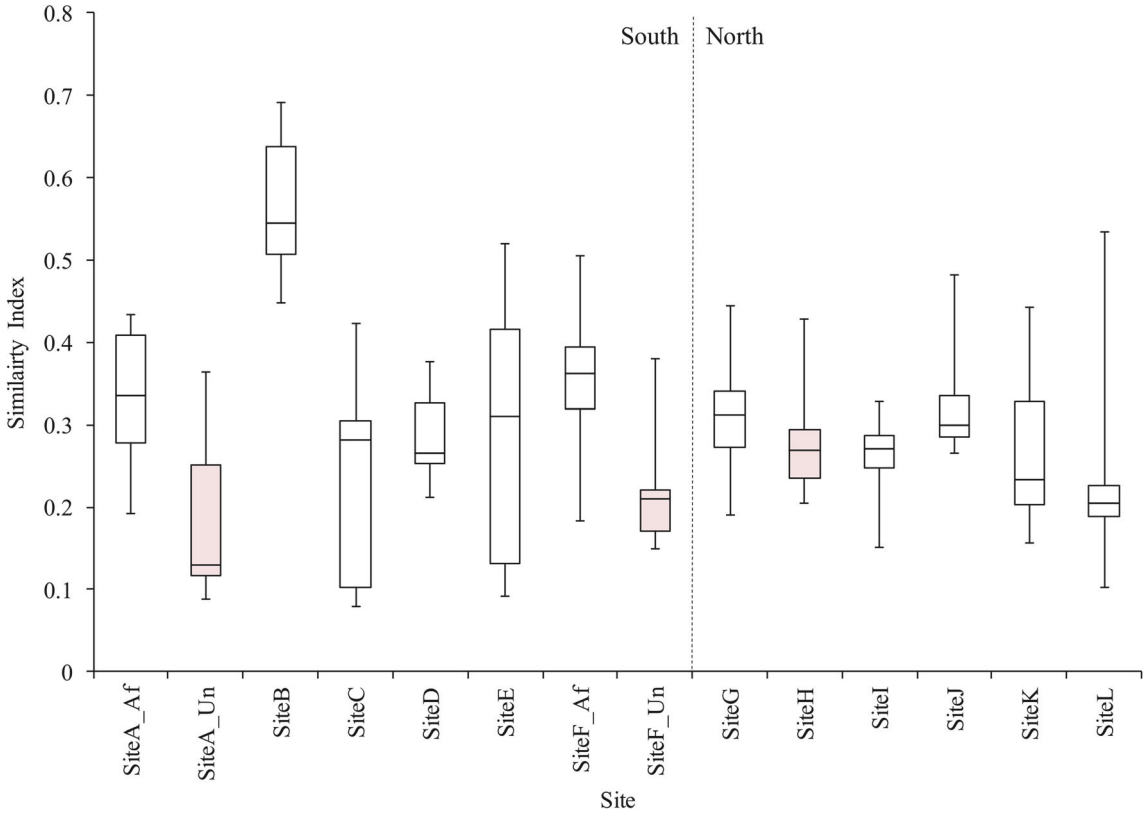


Fig. 2 Box and whisker plot of similarity indices calculated with a Bray-Curtis dissimilarity index. Each site code corresponds to those values listed in Table 1. As sites A and F contained both disease-affected and unaffected oyster stocks, these sites listed with the suffix '_Af" are disease-affected stocks, whilst those with '_Un' are unaffected stocks. Each site has five replicate oysters. Northern and southern samples are separated by the black vertical line. Disease-unaffected sites are shaded red

exhibited a microbiome composition that differed significantly from the disease-affected oysters in the northern region ($p = 0.0001$, $R = 0.48$; Fig. 3), with species diversity ($F_{(1, 48)} = 4.16$, $p = 0.047$) and richness ($F_{(1, 48)} = 14.15$, $p = 0.00046$) also differing between the northern and southern regions of the estuary, whilst species evenness did not differ

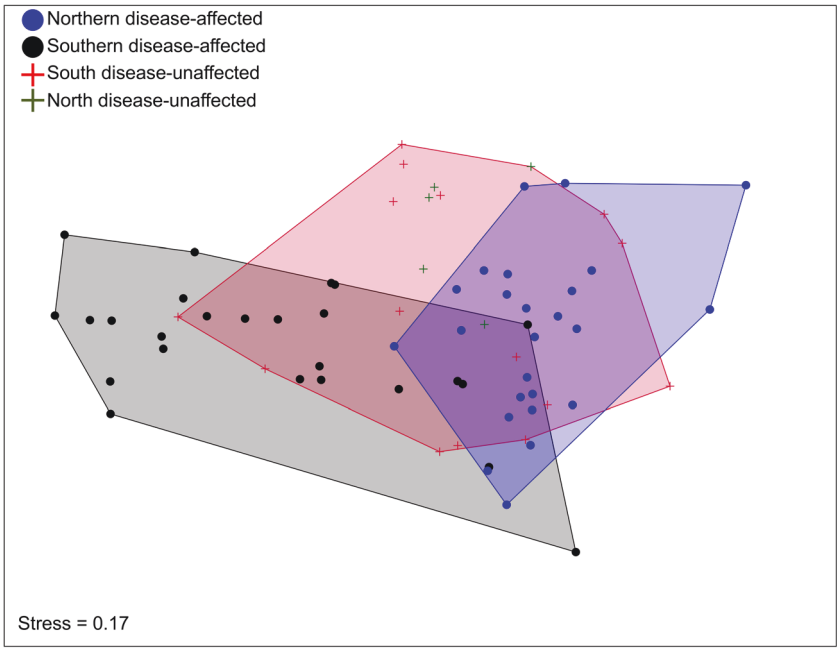


Fig. 3 nMDS plot showing spatial and disease state separation. Samples separate spatially based on region, as well as by their disease state. Axes 1 and 2 are plotted

between regions ($F_{(1,\ 48)} = 1.07$, $p = 0.31$) (Supplementary Table 1). Disease-affected hatchery sourced oysters also had a significantly different microbial assemblage to disease-affected wild sourced oysters ($p = 0.0052$, $R = 0.19$).

As we observed a significant difference between the disease-affected and disease-unaffected microbiome composition, we sought to examine persistent ('core') bacteria unique to these disease states and across the estuary as a whole (Supplementary Table 2). We identified 24 core members, of which 1 core OTU, an unidentified member of the *Mollicutes* class (OTU 89399), was unique to disease-affected microbiomes, whilst 8 OTUs were unique to disease-unaffected microbiomes (Supplementary Table 2). BLASTing the representative sequence for OTU 89399 against the NCBI database identified it as an uncultuered bacterium previously observed in *C. gigas* and another oyster species, *Saccostrea glomerata* (both BLAST hits were *E* value: 0, identity: 99%) [45, 46]. We then sought to examine which OTUs were driving the microbiome composition difference between disease states, and between spatial location. According to SIMPER analysis, the microbiomes of all unaffected samples (20 samples) were found to be 86.1% dissimilar to disease-affected samples in the south (25 samples) and 80.1% dissimilar to disease-affected samples in the north (25 samples; Supplementary Table 3).

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

In the south, the five most dominant OTUs across all sites were members of the *Brachyspiraceae* family (OTU 32677), *Spirochaetia* class (OTU 20129), *Mycoplasma* genus (OTU 38764), *Pseudoalteromonadaceae* family (OTU 18290) and the *Alphaproteobacteria* class (OTU 556), with these OTUs representing 10.8, 8.3, 7.8, 5.2 and 3.3% of sequences in the south. OTUs assigned to the *Pseudoalteromonadaceae* (OTU 18290) and *Brachyspiraceae* family (OTU 32677) were elevated in the disease-affected samples ($p = 0.48$ and $p = 0.00055$, respectively), whilst the remaining dominant OTUs were uniformly found in both unaffected and disease-affected samples.

Cromartys Bay *C. gigas* Microbiomes Shift in Accordance With Disease State

One location in Cromartys Bay (site A) contained both disease-affected (75% mortality) and unaffected (0% mortality) oyster stocks (Fig. 1). Due to the apparent role of spatial location in shaping the microbiome, and to account for any potentially confounding external factors such as cultivation method, hatchery source and unique local microenvironments, we focused on site A to examine differences in the microbiome of unaffected and disease-affected oysters. Whilst site F in Tilligerry Creek also had oyster stocks that were disease-affected and unaffected, this site was the only studied site to grow triploid oysters and information about the cultivation methods used at this site are unknown (Table 1). Because ploidy status and differing cultivation methods play a role in mortality outbreaks [15], site A was selected as the preferred site for comparative analysis.

All oysters from site A were cultivated in trays from hatchery spat that were acquired from the same source, thus minimising any potential differences in the microbiome caused by host genetics or cultivation method. Whilst these two sample groups displayed no difference in species diversity ($p = 0.46$) or evenness ($p = 0.84$) and only a marginally significant difference with species richness ($F_{(1,\ 8)} = 8.5$, $p = 0.019$), they exhibited significantly different microbiome composition ($p = 0.0073$, $R = 0.58$; Fig. 4, Supplementary Fig. 1 and Supplementary Table 1), and were 86.5% dissimilar to each other according to a SIMPER analysis. This dissimilarity identified with a SIMPER analysis was primarily driven by an over-representation of OTUs assigned to the *Pseudoalteromonadaceae* family (OTU 18290), *Bacteroidales* order (OTU 27418), JTB215 family (of the order *Clostridiales*; OTU 100999), *Bacteroidales* order (OTU 86667), *Paludibacter* genus (OTU 31616) and the *Bacteroides* genus (OTU 94495) in the disease-affected samples ($p = 0.0090$, $p = 0.0082$, $p = 0.0053$, $p = 0.0053$, $p = 0.019$, $p = 0.034$, respectively; Supplementary Table 4).

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

38778; $p = 0.016$), *Mycoplasma* genus (OTU 109572; $p = 0.030$), *Costertonia aggregata* (OTU 16511; $p = 0.025$) and the *Amphritea* genus (OTU 69264; $p = 0.05$) were significantly overrepresented in disease-unaffected samples (Table 2).

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

Discussion

Oyster Microbiomes Have Large Within-Site Heterogeneity

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

Oyster Microbiomes Are Influenced by Spatial Location

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

Within-Site Comparison of Microbiomes Between Disease States

As the site at Cromartys Bay (site A) contained oysters from both disease-affected and unaffected trays, we could remove the confounding effects of spatial variation to examine the within-site differences in the microbiome of disease-affected and disease-unaffected oysters. At site A, OTUs belonging to *V. harveyi* and an unidentified member of the *Vibrio* genus (Table 2) were found to be significantly more abundant in disease-affected samples. This is consistent with previous studies that have implicated the *Vibrio* community for their role in oyster disease outbreaks [7, 31]. *C. gigas* experimentally infected with a virulent *Vibrio* strain show an increase in *Vibrio* abundance in the microbiome, but the relative abundance remains low, despite significant disease symptoms and shifts in the rest of the microbiome [27]. Similarly, a small non-significant fold increase (~1.4) of *Vibrio* spp. abundance in heat stress corals is sufficient to cause large microbiome metabolic shifts [47]. Replacement of non-virulent strains with virulent strains has been documented in *C. gigas* during a summer mortality disease event [31], indicating that rare *Vibrio* species can disrupt the microbiome to a disease susceptible state. Whilst 16S rRNA is often unable to provide sufficient resolution to observe shifts within *Vibrio* populations, we observed increases in *V. harveyi*. This species has previously been identified as a *C. gigas* pathogen [48], and its increase in relative abundance in our sequencing data is consistent with the higher *Vibrio* bacterial counts in disease-affected samples from this specific site [17]. Furthermore, a prior study has demonstrated that the haemolymph microbiomes of *Vibrio*-infected *C. gigas* showed an increase in bacteria from potentially pathogenic genera, such as *Photobacterium*, and bacteria belonging to *Bacteroidia*, *Clostridia*, *Propionigenium*, *Vibrio*, *Arcobacter* and *Mollicutes* [27]. It is notable that similar increases in bacteria

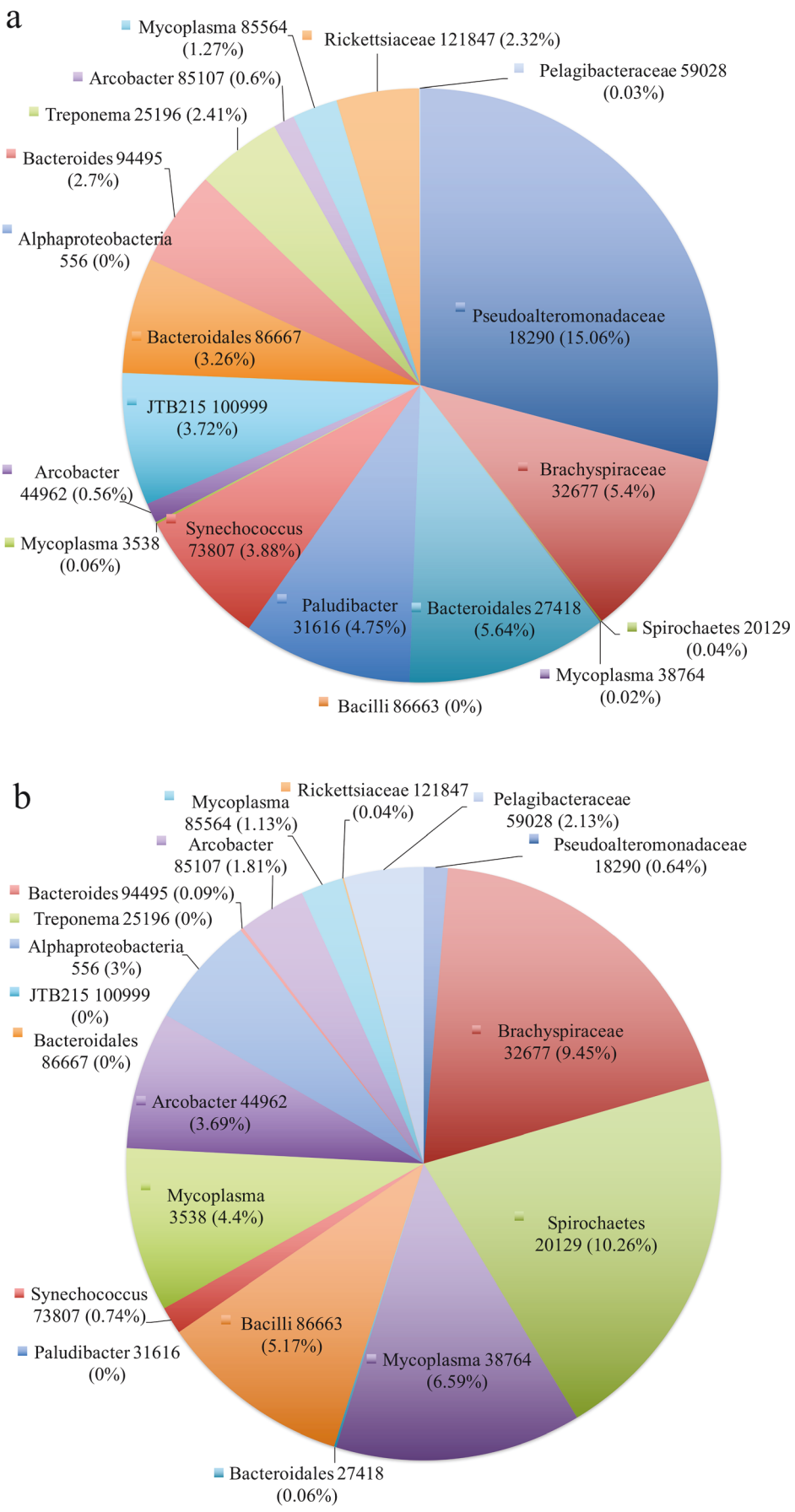


Fig. 4 Average abundance of Pacific oyster microbiomes at Cromartys Bay site A. **a** represents disease-affected samples, whilst **b** shows unaffected samples. A large shift in the microbiome is evident in disease-affected samples. OTUs representing less than 0.1% relative abundance were filtered out of the data set and only the top 20 OTUs are displayed, representing 51.7 and 49.2% of the averaged relative abundance community in **a** and **b**, respectively. Numbers in brackets on the pie chart represent the average relative abundance for each pie slice

Table 2 Significantly elevated OTUs (Kruskal-Wallis ANOVA; $p < 0.05$) between disease states at site A, as well as their average abundance

OTU	Diseased abundance (%)	Healthy abundance (%)
Bacteroides 94495	2.70	0.087
Paludibacter 31616	4.75	0
Costertonia aggregata 16511	0	0.18
Formosa crassostrea 88998	0.024	0
Tenacibaculum 125471	0	0.029
Fusibacter 119674	0.028	0
Fusobacterium 6434	0.57	0
Psychrilyobacter 42830	0.038	0
Nautella 120088	0.52	0.027
Octadecabacter antarcticus 25878	0.15	0
Desulfotalea 96648	0.069	0
Arcobacter 4188	1.70	0
Amphritea 69264	0	0.099
Pseudoalteromonas 38778	0	0.56
Pseudoalteromonas piscicida 110272	0	0.036
Vibrio harveyi 67592	0.15	0
Vibrio 122517	0.22	0
Treponema 25196	2.41	0
Mycoplasma 109572	0	0.36

OTUs assigned down to the genus or species level were chosen

belonging to these groups were observed in this study, with an unidentified member of the *Mollicutes* identified as being a core member of disease-affected microbiomes. Whilst it is not possible to determine to what extent these *Vibrio* OTUs caused this oyster mortality event, our observations of their elevated abundance in diseased oysters and evidence from previous work [7, 27, 31] point towards a potential role in infection or opportunistic colonisation.

Sewage-associated bacteria were found to be significantly elevated in disease-affected samples at site A; in particular, the *Paludibacter* genus (OTU 31616) was found to be completely absent in the unaffected samples, whilst those assigned to the *Bacteroides* genus (OTU 94495) were significantly elevated in disease-affected samples at both site A and in the southern region as a whole. The *Paludibacter* genus has been associated with animal waste [49, 50], whilst the *Bacteroides* genus is found to be heavily abundant in the human gastrointestinal tract (GIT) (reviewed by [51]) and can be used as an indicator for human faecal contamination [49, 52]. This over-representation of sewage/faecal associated OTUs in the southern site may be explained by the geographical features of the Port Stephens estuary, with Tilligerry creek having a strong agricultural and mining industrial presence on the creek, as well as being exposed to effluent off-flow from septic systems during periods of high rainfall [34]. Interestingly, there was a small rainfall event of 12.6 mm approximately 8 days before the start of the first mortalities at Cromartys Bay. Tilligerry creek flows out towards the northeast [34], as Cromartys Bay sits at the mouth of Tilligerry creek; it is possible that water entering Cromartys Bay influenced the bacterial communities in that bay as well. Due to their elevation in only the disease-affected samples, it may be possible that unaffected oysters had a greater capability to flush out these bacteria from their tissues.

Conclusion

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

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

References

1. Friedman CS, Estes RM, Stokes NA, Burge CA, Hargove JS, Barber BJ, Elston RA, Burreson EM, Reece KS (2005) Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. Dis Aquat Org 63:33–41. https://doi.org/10.3354/dao063033
2. Soletchnik P, Lambert C, Costil K (2005) Summer mortality of *Crassostrea gigas* (Thunberg) in relation to environmental rearing conditions. J Shellfish Res 24:197–207
3. Burge CA, Judah LR, Conquest LL, Griffin FJ, Cheney DP, Suhrbier A, Vadopalas B, Olin PG, Renault T, Friedman CS (2007) Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: the influence of oyster stock, planting time, pathogens, and environmental stressors. J Shellfish Res 26:163–172. https://doi.org/10.2983/0730-8000(2007)26[163:SSMOTP]2.0.CO;2
4. Soletchnik P, Ropert M, Mazurié J, Gildas Fleury P, Le Coz F (2007) Relationships between oyster mortality patterns and environmental data from monitoring databases along the coasts of France. Aquaculture 271:384–400. https://doi.org/10.1016/j.aquaculture.2007.02.049
5. Watermann BT, Herlyn M, Daehne B, Bergmann S, Meemken M, Kolodzey H (2008) Pathology and mass mortality of Pacific oysters, C*rassostrea gigas* (Thunberg), in 2005 at the east Frisian coast, Germany. J Fish Dis 31:621–630. https://doi.org/10.1111/j.1365-2761.2008.00953.x
6. Mortensen S, Strand A, Bodvin T, Alfjorden A, Skar CK, Jelmert A, Aspán A, Sælemyr L, Naustvoll LJ, Albretsen J (2016) Summer mortalities and detection of ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. Dis Aquat Org 117:171–176. https://doi.org/10.3354/dao02944
7. Garnier M, Labreuche Y, Garcia C, Robert M, Nicolas JL (2007) Evidence for the involvement of pathogenic bacteria in summer mortalities of the pacific oyster *Crassostrea gigas*. Microb Ecol 53:187–196. https://doi.org/10.1007/s00248-006-9061-9
8. Malham SK, Cotter E, O'Keeffe S, Lynch S, Culloty SC, King JW, Latchford JW, Beaumont AR (2009) Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: the influence of temperature and nutrients on health and survival. Aquaculture 287:128–138. https://doi.org/10.1016/j.aquaculture.2008.10.006
9. Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res 153:92–99. https://doi.org/10.1016/j.virusres.2010.07.011
10. Jenkins C, Hick P, Gabor M, Spiers Z, Fell SA, Gu X, Read A, Go J, Dove M, O'Connor W, Kirkland PD, Frances J (2013) Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis Aquat Org 105:109–126. https://doi.org/10.3354/dao02623
11. Petton B, Pernet F, Robert R, Boudry P (2013) Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. Aquac Environ Interact 3:257–273
12. Renault T, Bouquet AL, Maurice J-T, Lupo C, Blachier P (2014) Ostreid herpesvirus 1 infection among Pacific oyster (*Crassostrea gigas*) spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. Appl Environ Microbiol 80:5419–5426. https://doi.org/10.1128/AEM.00484-14
13. Jeffries VE (1982) Three Vibrio strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. Aquaculture 29:201–226. https://doi.org/10.1016/0044-8486(82)90136-3
14. Waechter M, Le Roux F, Nicolas JL, Marissal E, Berthe F (2002) Characterisation of *Crassostrea gigas* spat pathogenic bacteria. C R Biol 325:231–238. https://doi.org/10.1016/S1631-0691(02)01428-2
15. Pernet F, Barret J, Le Gall P, Corporeau C, Dégremont L, Lagarde F, Pépin JF, Keck N (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquac Environ Interact 2:215–237. https://doi.org/10.3354/aei00041
16. 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. doi: https://doi.org/10.3389/fmicb.2015.00686
17. Go J, Deutscher A, Spiers Z, Dahle K, Kirkland P, Jenkins C (2017) An investigation into mass mortalities of unknown aetiology in Pacific oysters, *Crassostrea gigas*, in port Stephens, new South Wales, Australia. Dis Aquat Org 125:227–242. https://doi.org/10.3354/dao03146
18. Lipovsky VP, Chew KK (1972) Mortality of Pacific oysters (*Crassostrea gigas*): the influence of temperature and enriched seawater on oyster survival. Proc Nat Shellfish Ass:72–82
19. Mori K (1979) Effects of artificial eutrophication on the metabolism of the Japanese oyster *Crassostrea gigas*. Mar Biol 53:361–369. https://doi.org/10.1007/bf00391619
20. Cotter E, Malham SK, O'Keeffe S, Lynch SA, Latchford JW, King JW, Beaumont AR, Culloty SC (2010) Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: the influence of growth, biochemistry and gametogenesis. Aquaculture 303:8–21. https://doi.org/10.1016/j.aquaculture.2010.02.030
21. Samain JF, Dégremont L, Soletchnik P, Haure J, Bédier E, Ropert M, Moal J, Huvet A, Bacca H, Van Wormhoudt A, Delaporte M, Costil K, Pouvreau S, Lambert C, Boulo V, Soudant P, Nicolas JL, Le Roux F, Renault T, Gagnaire B, Geret F, Boutet I, Burgeot T, Boudry P (2007) Genetically based resistance to summer mortality in the Pacific oyster (Crassostrea gigas) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture 268:227–243. https://doi.org/10.1016/j.aquaculture.2007.04.044
22. Segarra A, Mauduit F, Faury N, Trancart S, Dégremont L, Tourbiez D, Haffner P, Barbosa-Solomieu V, Pépin J-F, Travers M-A, Renault T (2014) Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. BMC Genomics 15:1–13. https://doi.org/10.1186/1471-2164-15-580
23. Dégremont L, Tanguy G, Delphine T, Jean-François P (2013) Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles? Aquaculture 408–409:51–57. https://doi.org/10.1016/j.aquaculture.2013.05.025
24. Dégremont L (2011) Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture 317:94–98. https://doi.org/10.1016/j.aquaculture.2011.04.029
25. Lang RP, Langdon C, Taris N, Camara M (2010) Use of laboratory assays to predict subsequent growth and survival of Pacific oyster

(*Crassostrea gigas*) families planted in coastal waters. Aquaculture 306:68–79. https://doi.org/10.1016/j.aquaculture.2010.04.023
26. Wegner KM, Volkenborn N, Peter H, Eiler A (2013) Disturbance induced decoupling between host genetics and composition of the associated microbiome. BMC Microbiol 13:252. https://doi.org/10.1186/1471-2180-13-252
27. Lokmer A, Wegner KM (2015) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J 9:670–682. https://doi.org/10.1038/ismej.2014.160
28. Lokmer A, Kuenzel S, Baines JF, Wegner KM (2016) The role of tissue-specific microbiota in initial establishment success of Pacific oysters. Environ Microbiol 18:970–987. https://doi.org/10.1111/1462-2920.13163
29. Trabal N, Mazon-Suastegui JM, Vazquez-Juarez R, Asencio-Valle F, Morales-Bojorquez 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 64:555–569. https://doi.org/10.1007/s00248-012-0039-5
30. Trabal Fernández N, Mazón-Suástegui JM, Vázquez-Juárez R, Ascencio-Valle F, Romero J (2014) Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis, Crassostrea gigas and Crassostrea sikamea*) during commercial production. FEMS Microbiol Ecol 88:69–83. https://doi.org/10.1111/1574-6941.12270
31. 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 9:1523–1531. https://doi.org/10.1038/ismej.2014.233
32. Roy PS, Williams RJ, Jones AR, Yassini I, Gibbs PJ, Coates B, West RJ, Scanes PR, Hudson JP, Nichol S (2001) Structure and function of south-east Australian estuaries. Estuar Coast Shelf Sci 53:351–384. https://doi.org/10.1006/ecss.2001.0796
33. Australian Bureau of Statistics (2016) Port Stephens census data (Statistical area). http://quickstats.censusdata.abs.gov.au/census_services/getproduct/census/2016/quickstat/10603?opendocument. Accessed 1 May 2018
34. Earth Tech Engineering Pty Ltd (2008) Tilligerry creek management plan. https://www.portstephens.nsw.gov.au/__data/assets/pdf_file/0020/9380/FINAL_TilligerryCreekManagmentPlan_Aug_2008_reduced.pdf. Accessed 6 November 2017
35. Lokmer A, Goedknegt MA, Thieltges DW, Fiorentino D, Kuenzel S, Baines JF, Wegner KM (2016) Spatial and temporal dynamics of Pacific oyster hemolymph microbiota across multiple scales. Front Microbiol 7:1367. https://doi.org/10.3389/fmicb.2016.01367
36. Vezzulli L, Stagnaro L, Grande C, Tassistro G, Canesi L, Pruzzo C (2017) Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). Microb Ecol 75:495–504. https://doi.org/10.1007/s00248-017-1051-6
37. 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 21:39–43. https://doi.org/10.1051/alr:2008009
38. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, pp 115–175
39. Turner S, Pryer KM, Miao VP, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46:327–338
40. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303
41. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461
42. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610–618. https://doi.org/10.1038/ismej.2011.139
43. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. https://doi.org/10.1128/aem.00062-07
44. Hammer Ø, Harper DAT, Ryan PD (2001) Past: Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:XIX–XXX
45. Fernandez-Piquer J, Bowman JP, Ross T, Tamplin ML (2012) Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure. J Appl Microbiol 112:1134–1143. https://doi.org/10.1111/j.1365-2672.2012.05287.x
46. Green TJ, Barnes AC (2010) Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. J Appl Microbiol 109:613–622. https://doi.org/10.1111/j.1365-2672.2010.04687.x
47. Thurber RV, Willner-Hall D, Rodriguez-Mueller B, Desnues C, Edwards RA, Angly F, Dinsdale E, Kelly L, Rohwer F (2009) Metagenomic analysis of stressed coral holobionts. Environ Microbiol 11:2148–2163. https://doi.org/10.1111/j.1462-2920.2009.01935.x
48. 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 59:787–798. https://doi.org/10.1007/s00248-009-9620-y
49. Newton RJ, VandeWalle JL, Borchardt MA, Gorelick MH, McLellan SL (2011) *Lachnospiraceae* and *Bacteroidales* alternative fecal indicators reveal chronic human sewage contamination in an Urban Harbor. Appl Environ Microbiol 77:6972–6981. https://doi.org/10.1128/AEM.05480-11
50. Ueki A, Akasaka H, Suzuki D, Ueki K (2006) *Paludibacter propionicigenes* gen. Nov., sp. nov., a novel strictly anaerobic, gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. Int J Syst Evol Microbiol 56:39–44. https://doi.org/10.1099/ijs.0.63896-0
51. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307:1915–1920. https://doi.org/10.1126/science.1104816
52. Kreader CA (1995) Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl Environ Microbiol 61:1171–1179

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 March 2019
doi: 10.3389/fmicb.2019.00473

Variability in the Composition of Pacific Oyster Microbiomes Across Oyster Families Exhibiting Different Levels of Susceptibility to OsHV-1 μvar Disease

*William L. King[1,2], Nachshon Siboni[2], Nathan L. R. Williams[1], Tim Kahlke[2], Khue Viet Nguyen[1,2], Cheryl Jenkins[3], Michael Dove[4], Wayne O'Connor[4], Justin R. Seymour[2] and Maurizio Labbate[1]**

[1] The School of Life Sciences, University of Technology Sydney, Ultimo, NSW, Australia, [2] Climate Change Cluster, University of Technology Sydney, Ultimo, NSW, Australia, [3] NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, NSW, Australia, [4] NSW Department of Primary Industries, Port Stephens Fisheries Institute, Port Stephens, NSW, Australia

OPEN ACCESS

Edited by:
Sebastian Fraune, University of Kiel, Germany

Reviewed by:
Mathias Wegner, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research (AWI), Germany
Eve Toulza, Université de Perpignan Via Domitia, France

***Correspondence:**
Maurizio Labbate
maurizio.labbate@uts.edu.au

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *01 November 2018*
Accepted: *22 February 2019*
Published: *11 March 2019*

Citation:
King WL, Siboni N, Williams NLR, Kahlke T, Nguyen KV, Jenkins C, Dove M, O'Connor W, Seymour JR and Labbate M (2019) 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. doi: 10.3389/fmicb.2019.00473

Oyster diseases are a major impediment to the profitability and growth of the oyster aquaculture industry. In recent years, geographically widespread outbreaks of disease caused by ostreid herpesvirus-1 microvariant (OsHV-1 μvar) have led to mass mortalities among *Crassostrea gigas*, the Pacific Oyster. Attempts to minimize the impact of this disease have been largely focused on breeding programs, and although these have shown some success in producing oyster families with reduced mortality, the mechanism(s) behind this protection is poorly understood. One possible factor is modification of the *C. gigas* microbiome. To explore how breeding for resistance to OsHV-1 μvar affects the oyster microbiome, we used 16S rRNA amplicon sequencing to characterize the bacterial communities associated with 35 *C. gigas* families, incorporating oysters with different levels of susceptibility to OsHV-1 μvar disease. The microbiomes of disease-susceptible families were significantly different to the microbiomes of disease-resistant families. OTUs assigned to the *Photobacterium*, *Vibrio*, *Aliivibrio*, *Streptococcus,* and *Roseovarius* genera were associated with low disease resistance. In partial support of this finding, qPCR identified a statistically significant increase of *Vibrio*-specific 16S rRNA gene copies in the low disease resistance families, possibly indicative of a reduced host immune response to these pathogens. In addition to these results, examination of the core microbiome revealed that each family possessed a small core community, with OTUs assigned to the *Winogradskyella* genus and the *Bradyrhizobiaceae* family consistent members across most disease-resistant families. This study examines patterns in the microbiome of oyster families exhibiting differing levels of OsHV-1 μvar disease resistance and reveals some key bacterial taxa that may provide a protective or detrimental role in OsHV-1 μvar disease outbreaks.

Keywords: *Crassostrea gigas*, microbiome, ostreid herpesvirus, disease resistance, *Vibrio*, POMS

INTRODUCTION

The Pacific oyster, *Crassostrea gigas* is a globally cultivated oyster species, but the cultivation of this species has been increasingly impacted by disease events (Azéma et al., 2015). These disease events are largely caused by viral and bacterial etiological agents (Friedman et al., 2005; Garnier et al., 2007; Segarra et al., 2010; King et al., 2019), but in some instances no clear etiological agent is identifiable (Go et al., 2017; King et al., 2018). A major pathogen of *C. gigas* is the ostreid herpesvirus 1 (OsHV-1), and its micro variant form (OsHV-1 μvar) (Davison et al., 2005; Segarra et al., 2010). This virus has caused severe mortality outbreaks over the last two decades (Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Mortensen et al., 2016), with some outbreaks resulting in over 90% mortality and leading to the death of many millions of oysters (ASI, 2015).

To combat the impact of OsHV-1 μvar, a variety of approaches including modifying husbandry practices (e.g., increased oyster cultivation height) and breeding disease resistant oysters have been applied, with varying degrees of success (Dégremont, 2011; Paul-Pont et al., 2013; Whittington et al., 2015). Breeding programs generally involve breeding oyster genetic lines that have greater survival rates following exposure to OsHV-1 (Dégremont, 2011, 2013; Dégremont et al., 2015b; Camara et al., 2017). While these breeding programs have shown some success, resistant families still experience varying degrees of mortality (juvenile oysters 5–19%; larvae up to 86%) (Dégremont, 2011; Dégremont et al., 2013, 2016b), and the mechanism(s) underpinning resistance are often not easily distinguishable.

A number of studies have characterized the physiological and immunological factors driving OsHV-1 and OsHV-1 μvar resistance in selectively bred oysters (Sauvage et al., 2009; Dégremont, 2011, 2013; Azéma et al., 2015; Dégremont et al., 2015a, 2016a,b). Factors such as increased oyster size and weight are associated with increased resistance to infection, but why this occurs is currently unclear (Dégremont, 2013; Dégremont et al., 2015b). Other studies have determined that resistant oysters have a greater capacity to clear OsHV-1 from their tissues and supress virus replication (Dégremont, 2011; Segarra et al., 2014). When examined from an immunologic perspective, resistant oysters appear to have greater capacity to induce autophagy genes when infected by OsHV-1 compared to susceptible oysters (Moreau et al., 2015).

Another contributing factor that has received less attention is the oyster microbiome. A previous study has shown that despite being positive for OsHV-1 μvar, antibiotic-treated oysters displayed significantly reduced mortalities in comparison to untreated oysters (Petton et al., 2015). Furthermore, the total bacterial load, including the *Vibrio* community, is significantly elevated following OsHV-1 μvar infection and this elevation is necessary to cause mortality (de Lorgeril et al., 2018). *Vibrio* bacteria are commonly isolated from OsHV-1 infected oysters (Segarra et al., 2010; Garcia et al., 2011; Jenkins et al., 2013; Keeling et al., 2014), with a recent study concluding that OsHV-1 μvar infection causes immune-suppression of the oyster host, allowing opportunistic bacteria (including *Vibrio* species) to infect the oyster (de Lorgeril et al., 2018). In other organisms, studies have implicated the host microbiome as modulating the immune system, suggesting it is critical in host defense and overall health (reviewed by Shreiner et al., 2015) and in influencing host behavior (Shin et al., 2011).

To better understand how breeding for OsHV-1 μvar disease resistance affects the *C. gigas* microbiome and to elucidate whether specific taxa are associated with susceptibility and resistance, we examined the microbiome of 35 *C. gigas* families with varying degrees of disease-resistance. To remove the confounding effects of time and location, these oysters were deployed at a single location and sampled at the same time. In addition, the comparison of distinct *C. gigas* families provided the opportunity to determine whether they harbored distinct microbial community assemblages and whether persistent bacterial taxa (core microbiome) common across the families could be identified.

MATERIALS AND METHODS

Sources and Sampling of *C. gigas*

Australian Seafood Industries (ASI) is an oyster aquaculture industry-owned company that since the first OsHV-1 μvar outbreak in 2010 has been breeding *C. gigas* families for OsHV-1 μvar disease resistance through field exposure. In 2016, ASI deployed 35 (n = 35) 5th generation families (5 consecutive years of biparental breeding) of juvenile *C. gigas* into three areas known to harbor the OsHV-1 virus, the Georges River (NSW, Australia; 34.035S, 151.145E), Pipe Clay Lagoon (TAS, Australia; 42.970S, 147.525E) and Pittwater (TAS, Australia; 42.802S, 147.509E) (Kube et al., 2018). Based on these field disease-exposure studies, expected breeding values (EBVs) were calculated by ASI. These EBVs are an estimation of how well the oysters will perform for a particular trait and the likelihood of passing those traits to their progeny. For the purposes of this study, families were classified into 'resistance groups' (RG) based on their OsHV-1 μvar disease resistance EBV. Families with an EBV greater than 0.6 were placed into RG1 (high disease-resistance), those with an EBV greater than 0.3 and less than 0.6 were placed into RG2 (medium disease-resistance), and families with an EBV less than 0.3 were placed into RG3 (low disease-resistance) (**Table 1**). The estimated heritability is the likelihood of the offspring demonstrating a particular trait, in this case OsHV-1 μvar disease resistance. Resistance is determined by the combination of many genes, since the stock used are derived from a number of genetically distinct families, each family differs in its resistance, and crosses between families differ.

In addition to disease-resistance, EBVs of other oyster traits were also provided by ASI. These traits include: meat condition, the ratio of wet meat to the total weight; depth index, the ratio of shell depth to shell length; shell length; oyster weight, including the oyster shell; and width index, the ratio of shell width to shell length. As EBV's are proprietary information, rather than providing absolute values for each index, we generated a 'rank' system to categorize families according to each index, with ranks of 1 being the highest (**Table 1**).

TABLE 1 | Expected breeding value ranks for the studied oyster families including OsHV-1 μvar disease-resistance.

Family line	OsHV-1 μvar resistance	Resistance group (RG)	Meat condition	Depth index	Shell length	Oyster weight	Width index
F_01	8	RG2	22	6	28	7	7
F_02	25	RG3	20	1	29	10	10
F_03	6	RG2	17	18	21	13	13
F_07	16	RG2	6	4	34	1	1
F_10	26	RG3	16	10	32	5	5
F_11	24	RG3	11	7	31	6	6
F_15	29	RG3	6	4	34	1	1
F_16	31	RG3	3	14	11	23	23
F_19	17	RG2	17	18	21	13	13
F_20	28	RG3	4	20	9	13	13
F_23	15	RG2	13	28	19	19	19
F_25	20	RG2	6	21	19	20	20
F_26	32	RG3	12	8	25	22	22
F_27	22	RG3	25	2	27	17	17
F_29	7	RG2	27	31	2	35	35
F_30	18	RG2	23	30	3	33	33
F_35	34	RG3	1	27	10	8	8
F_36	10	RG2	9	24	17	24	24
F_37	12	RG2	4	9	30	11	11
F_39	27	RG3	2	15	17	9	9
F_40	11	RG2	17	12	26	21	21
F_43	19	RG2	20	10	21	28	28
F_51	23	RG3	14	28	13	12	12
F_61	30	RG3	14	35	1	34	34
F_62	33	RG3	24	24	7	17	17
F_65	13	RG2	30	31	3	30	30
F_66	1	RG1	30	31	3	30	30
F_67	9	RG2	30	31	3	30	30
F_68	3	RG1	30	16	15	25	25
F_69	5	RG1	30	16	15	25	25
F_72	2	RG1	35	21	13	16	16
F_77	4	RG1	26	24	8	25	25
F_80	21	RG3	29	23	12	29	29
F_84	14	RG2	28	2	33	3	3
F_86	35	RG3	10	13	24	4	4

Survival data was found after deployment in three different OsHV-1 μvar positive estuaries across Australia. Expected breeding values (EBVs), including OsHV-1 μvar disease-resistance, are shown as a rank number out of 35.

For this microbiome study, the families were deployed into the Georges River (34.035S, 151.145E) on the 16th of August 2016 and sampled 2 months after deployment date. The 2-month deployment time was the first opportunity to sample the deployed juvenile oysters and was sufficient time to ensure no evidence of disease or morbidity. Oysters were deployed in a resolvable incomplete block design to account for micro-geographic variation, blocks were subsections of a replicate and there were three replicates for each family, with each family stocked into a subsection of the tray (Kube et al., 2018). Five oysters from each of the 35 families (total = 175 samples) were sampled and immediately placed on ice and transported to the laboratory where they were stored at −80°C until further processing.

DNA Extraction, Sequencing, and Bioinformatics

The outer shell of the five sampled oysters was rinsed under running tap water to remove any remaining mud and debris. Defrosted oysters were then shucked with sterilized shucking knifes and approximately 25 mg of adductor muscle tissue was aseptically removed using sterile scalpel blades.

The Qiagen DNeasy blood and tissue kit (catalogue: 69506) was used to extract DNA samples, as per the manufacturer's instructions. Microbial community composition within samples was subsequently assessed using 16S rRNA amplicon sequencing, whereby the ribosomal 16S rRNA V1–V3 region was targeted using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′) primer pair. The PCR cycling conditions were as follows: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Amplicons were sequenced using the Illumina MiSeq platform (2 × 300 bp) using standard approaches (Ramaciotti Centre for Genomics at the University of New South Wales, Sydney, NSW, Australia). Raw data files in FASTQ format were deposited in the NCBI Sequence Read Archive (SRA) under the Bioproject number PRJNA497763.

Briefly, 16S rRNA paired-end DNA sequences were joined using Flash (Magoc and Salzberg, 2011) and subsequently trimmed using Mothur (Schloss et al., 2009) (Parameters: maxhomop = 5, maxambig = 0, minlength = 432, maxlength = 506). The resulting fragments were clustered into operational taxonomic units (OTUs) at 97% sequence identity, and chimeric sequences were identified using vsearch (Rognes et al., 2016). Taxonomy was assigned in QIIME (Caporaso et al., 2010) using the uclust algorithm (Edgar, 2010) against the Silva v128 database. Mitochondrial and chloroplast data were filtered out of the dataset and the remaining data were rarefied to allow for even coverage across all samples (**Supplementary Data Sheet 2**). OTUs representing less than 0.1% relative abundance in an individual sample were also filtered from the dataset (**Supplementary Tables 1, 2**).

Core Microbiome Analysis

To determine whether a core oyster microbiome could be characterized, we examined the microbiome of oysters at three different thresholds. First, for individual families, then for RGs, then for all samples together. A core OTU was defined as an OTU that was present in at least all but one replicate (to account for outliers) within a family. To achieve this, the panbiom.py script was used as detailed in Kahlke (2017). Briefly, the final biom file generated during the QIIME analysis was used in conjunction with a treatment file that identifies which samples are replicates within a family. The panbiom.py arguments were

as follows: a replicate threshold of 1 (-r parameter) and an outlier threshold of 'x' (-x parameter). The -x parameter treats the replicate threshold value as an outlier threshold value, simply put, it can be absent in one replicate sample (indicated by -r = 1 and -x = x).

Quantitative PCR (qPCR)

Due to the potential role of *Vibrio* in OsHV-1 μvar disease dynamics (Segarra et al., 2010; Jenkins et al., 2013; Lemire et al., 2015; Petton et al., 2015; de Lorgeril et al., 2018), quantitative PCR (qPCR) was used to examine patterns in *Vibrio* abundance across the RGs. qPCR was performed using an epMotion 5075l Automated Liquid Handling System on a Bio-Rad CFX384 Touch Real-Time PCR Detection System with a six-point calibration curve and negative controls on every plate. The calibration curve was built from a known amount of amplicon DNA measured by Qubit, followed by a 10-fold dilution to fill out the calibration curve. All sample analyses were performed with three technical replicates, using the following reaction mixture: 2.5 μL iTaq Universal SYBR Green supermix, 0.4 μM of each forward and reverse primer, 1 μL of diluted (1:15) template DNA, and the remainder made up with water. To quantify abundance of the *Vibrio* community, the *Vibrio*-specific 16S rRNA primers Vib1-f (5′-GGCGTAAAGCGCATGCAGGT-3′) and Vib2-r (5′-GAAATTCTACCCCCCTCTACAG-3′) were used (Thompson et al., 2004; Vezzulli et al., 2011; Siboni et al., 2016). The qPCR cycling conditions were as follows: 95°C for 3 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The resulting data were normalized to both elution volume (200 μL) and tissue weight. A coefficient of variation (CV) was then calculated for the technical triplicates, and samples with CV > 10% were removed from the analysis. A melting curve was added to the end of every run to confirm the presence of a single PCR product.

Statistical Analysis

Comparisons of alpha diversity were performed with a one-way ANOVA followed by a Tukey's pairwise test. Normalized [square root (x)] data were used to compare community compositions using a non-metric multidimensional scaling analysis (nMDS) with a Bray–Curtis similarity index. To determine significantly different microbial assemblage between families and RGs, and to compare qPCR data, a one-way PERMANOVA was used. To examine which OTUs contributed to differences between RGs, a SIMPER analysis with a Bray–Curtis similarity index was used. To define associations between breeding values and OTUs, breeding values were normalized (x-mean/standard deviation) and used within a canonical correspondence analysis (CCA). All analyses were performed using the PAST statistical software (Hammer et al., 2001). To determine whether an OTU was significantly elevated in a particular RG, the group_significance.py script using the default analysis (Kruskal–Wallis ANOVA) was used in QIIME. To examine correlations between EBVs, we performed a maximal information-based non-parametric exploration (MINE) analysis (Reshef et al., 2011).

RESULTS

The *C. gigas* Microbiome

Following data filtering and rarefication, a total of 3294 OTUs were observed across the entire dataset, and of these, 68.5% occurred at below 1% of the total relative abundance. Conversely, across all samples and spanning all RGs, a member of the *Pseudomonas* genus (OTU 2034) was found to be the most relatively abundant OTU comprising 5.6% of the bacterial community. This was followed by OTUs matching an uncultured bacterium in the *Psychrobacter* genus (OTU 1488) and an uncultured bacterium in the *Mycoplasma* genus (OTU 3150), which represented 4.8 and 4% of the *C. gigas* microbiome across the whole dataset respectively (**Figure 1**).

Variability in the *C. gigas* Microbiome Across Different Resistance Lines

To determine whether breeding for disease resistance influences the *C. gigas* microbiome, the microbiome of oysters assigned to RGs were characterized and compared. Alpha diversity, quantified using Shannon's diversity index was significantly higher in RG2 when compared to the RG3 RG ($F_{(1,141)}$ = 6.8, p = 0.025), but did not vary significantly when compared to RG1 ($F_{(1,93)}$ = 0.4, p = 0.51). Species richness (Chao1) did not differ significantly between any of the RGs (RG1 vs. RG2 – $F_{(1,93)}$ = 0.03, p = 0.85; RG1 vs. RG3 – $F_{(1,94)}$ = 1.3, p = 0.26; RG2 vs. RG3 – $F_{(1,141)}$ = 1.08, p = 0.30).

Comparisons of microbiome composition (beta diversity) across different RGs revealed that the microbiomes of RG1 and RG2 were both significantly different to the least disease resistant group, RG3 (p = 0.019 and p = 0.0001; $F_{(1,94)}$ = 1.47 and $F_{(1,141)}$ = 2.93 respectively). No significant difference was found between the microbiomes of RG1 and RG2 ($F_{(1,93)}$ = 1.29, p = 0.055). Statistical comparisons between RG2 and RG3 appeared to be stronger than those between RG1 and RG3, possibly due to more families being assigned to RG2, therefore potentially adding more microbiome variability to this group. No clear dissimilarity in the microbiome of the RGs was apparent in a 3D nMDS (Stress = 0.34), or a PCoA (**Supplementary Figure 1**). SIMPER comparisons showed that the composition of the microbiomes associated with RG1 and RG2 were 81.83 and 82.12% dissimilar to RG3 respectively (**Supplementary Tables 3, 4**).

As the RG with the lowest level of disease-resistance (RG3) was found to have a significantly different microbial assemblage to both RG2 and RG1, we examined which OTUs were responsible for driving the differences in microbiome structure between these groups (**Figure 2**). An OTU assigned to the *Pseudomonas* genus (OTU 2034; the most abundant OTU in the entire dataset) was over-represented in the RG3 microbiome relative to both RG1 ($H_{(1,94)}$ = 7.6, p = 0.0058) and RG2 ($H_{(1,141)}$ = 15, p = 0.00011). Conversely, an OTU assigned to the *Tenacibaculum* genus (OTU 2636) and two separate OTUs assigned to the *Dokdonia* genus (OTUs 2162 and 1526) were all significantly under-represented in RG3 (*Tenacibaculum* RG1 $H_{(1,94)}$ = 4.5, p = 0.033 and RG2

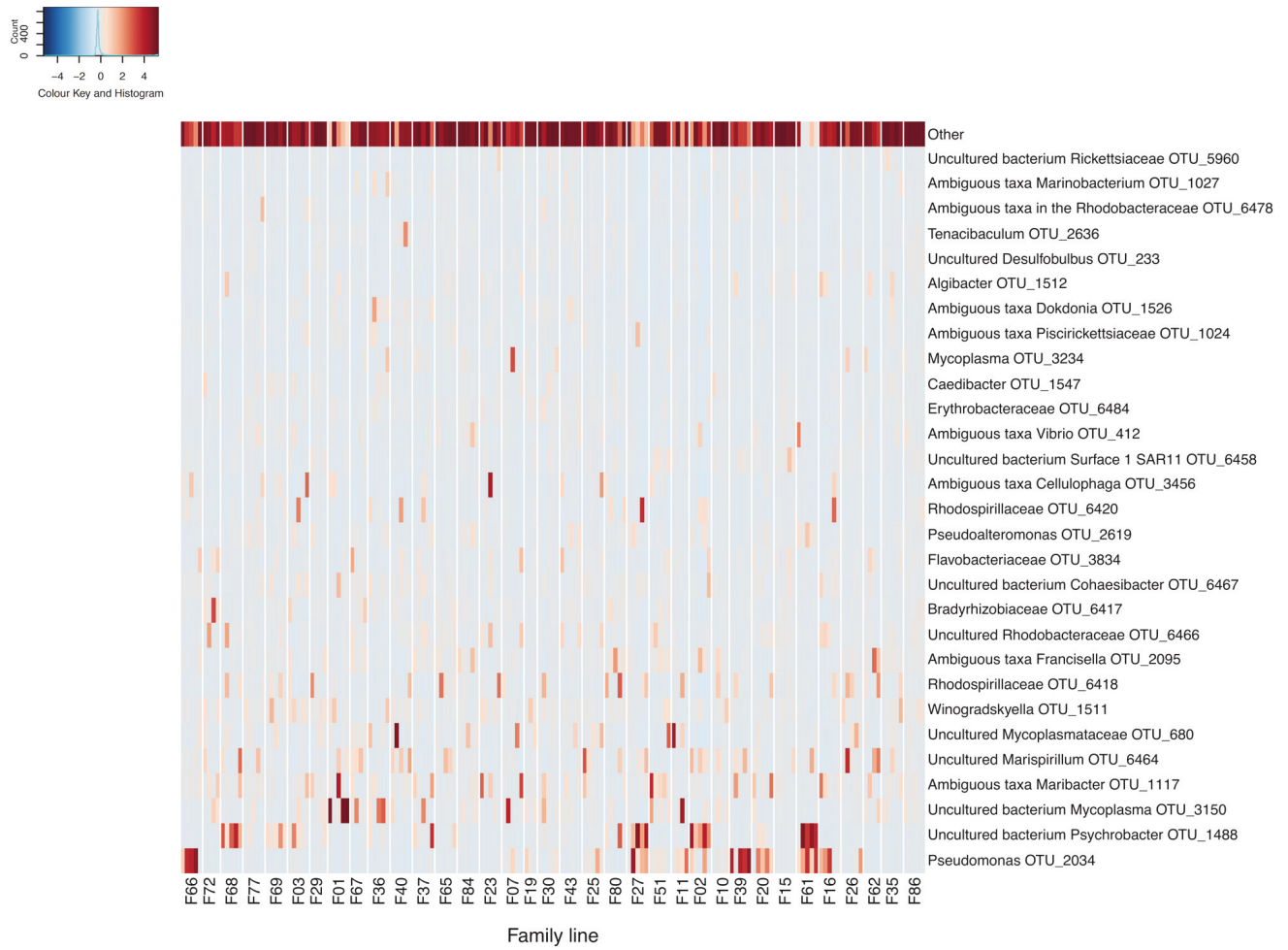


FIGURE 1 | Heatmap of scaled OTU relative abundance for the 30 most abundant OTUs, as well as the remaining summed lowly abundant OTUs. Families ordered by OsHV-1 µvar disease-resistance. Heatmap was made using the R statistical environment using scaled data with the gplots and RColorBrewer packages (Neuwirth, 2014; Warnes et al., 2016; R Core Team, 2017).

$H_{(1,141)} = 15.2, p = 0.0056$; *Dokdonia* RG1 $H_{(1,94)} = 7.7, p = 0.0001$ and RG2 $H_{(1,141)} = 30.3, p < 0.0001$).

Notably, a member of the *Vibrio* genus (OTU 412) was found to be significantly over-represented in the least disease-resistant group (RG3) relative to the most disease-resistant group (RG1) ($H_{(1,94)} = 4.4$, $p = 0.036$). Due to the previously demonstrated importance of *Vibrio* species in OsHV-1 µvar infection (Segarra et al., 2010; Jenkins et al., 2013; de Lorgeril et al., 2018), we subsequently employed a *Vibrio*-specific 16S rRNA qPCR assay to compare total abundances of *Vibrio* across RGs. A significant elevation of *Vibrio* 16S rRNA gene copies was observed in RG3 compared to RG1 ($F_{(1,94)} = 2.86$, $p = 0.027$) and RG2 ($F_{(1,141)} = 3.25$, $p = 0.014$) (average of 179, 107, and 75 gene copies mg of tissue^{-1} respectively; **Supplementary Figure 2**). Furthermore, OTUs assigned to the *Vibrio* genus were significantly elevated in RG3 when compared to RG1 ($F_{(1,94)} = 4.27, p = 0.011$), but not RG2 ($F_{(1,141)} = 2.48, p = 0.07$). To determine the extent of whether *Vibrio* OTUs were driving the differences between RG1 and RG3 microbiomes, OTUs assigned to the *Vibrio* genus were removed and the RG beta diversity comparison was reperformed. When doing this, we observed a slight weakening of the statistical comparison between RG1 and RG3, from ($F_{(1,94)} = 1.47, p = 0.019$) to ($F_{(1,94)} = 1.46, p = 0.024$).

A CCA was used to highlight associations between specific OTUs, OsHV-1 µvar disease-resistance and EBVs of other traits (**Figure 3**). OTUs matching the *Cupriavidus* (OTU 2182) and *Psychrilyobacter* (OTU 5046) genera were closely coupled with disease-resistance, followed by a member of the *Tenacibaculum* (OTU 2153) genus and an uncultured bacterium in the *Frankiales* order (OTU 5180). While OTUs assigned to members of the *Photobacterium* (OTU 1063; OTU 654; OTU 1053), *Vibrio* (OTU 651; OTU 653) and *Aliivibrio* (OTU 1248) genera were negatively associated with disease-resistance, but strongly associated with meat condition. Furthermore, members of the *Streptococcus* (OTU 814) and *Roseovarius* (OTU 7180) genera were closely associated with depth and width index, and also negatively associated with disease-resistance. The community composition was largely influenced by the

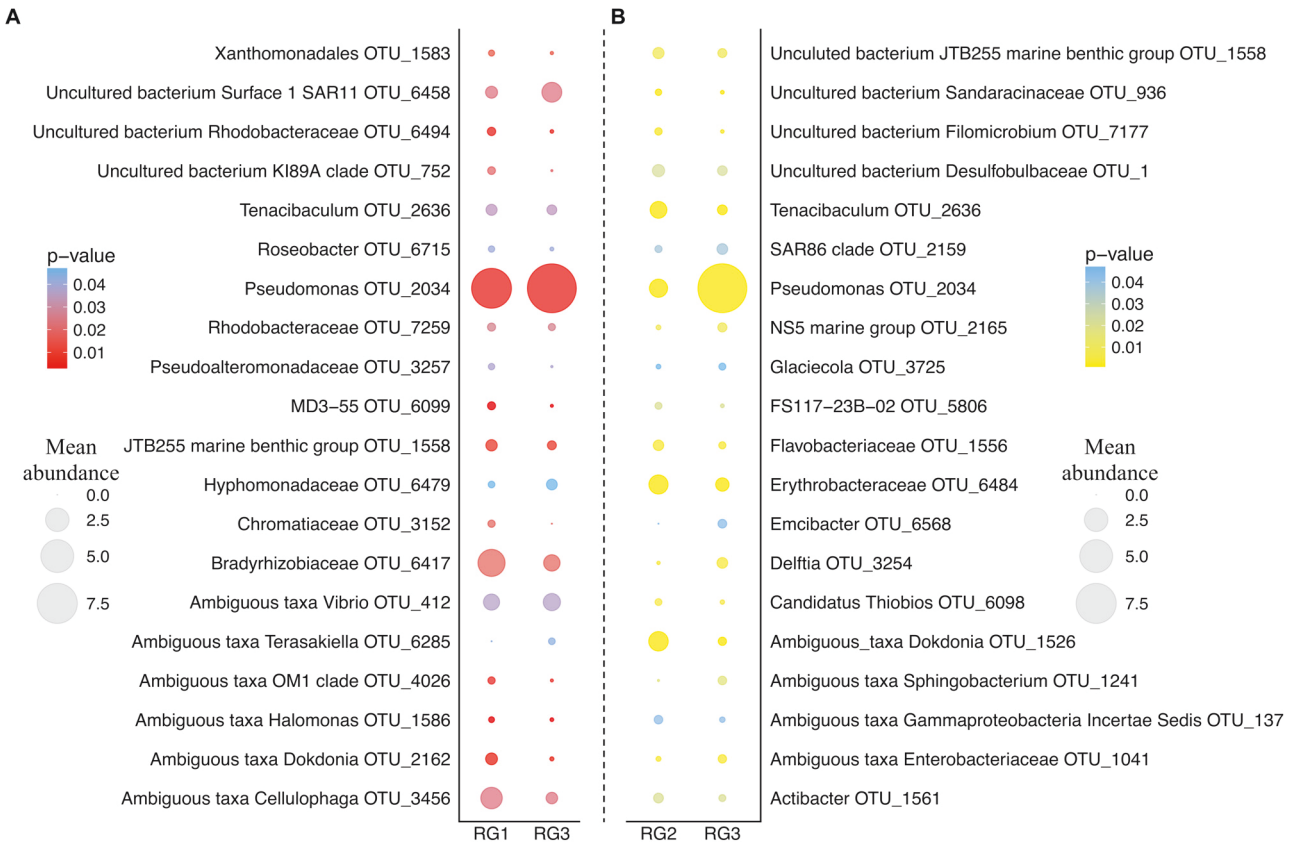


FIGURE 2 | Bubble plot of group_significance.py analysis results using the default Kruskal–Wallis parameters. **(A)** Represents the comparison between RG1 and RG3. **(B)** Represents the comparison between RG2 and RG3. Color represents the strength of the *p*-value. Size represents the mean relative abundance of that OTU across the whole resistance group (RG). OTUs assigned to the genus and species level were chosen, and those 20 most abundant from each RG are displayed.

first axis, driven by growth related EBVs. A MINE analysis identified a negative correlation between disease resistance and width index (p = 0.047; linear regression = −0.34), and a positive correlation between disease resistance and oyster weight (p = 0.038; linear regression = 0.15). Shell length and depth index had the strongest negative correlation (p = < 0.001; linear regression = −0.92), while oyster weight and shell length had the strongest positive correlation (p = 0.002; linear regression = 0.74; **Supplementary Table 5**).

Defining the Core *C. gigas* Microbiome Across Different Resistance Lines

Due to the dynamic nature of oyster microbiomes, identifying a core microbiome can provide insights into which members may be driving the within-microbiome interactions and possibly shaping the community composition. While we were unable to identify a universal core microbiome across all samples, analyses of individual families revealed that each family had a small core microbiome (9–109 OTUs), with many of these OTUs shared across families. Families 30 and 84, within RG2, shared the most core OTUs (4) (**Supplementary Figure 3**). In contrast, family 19 of RG2 had the most unique core OTUs (27), that is those core OTUs not shared with any other family. To determine how many unique core OTUs were present in each oyster family (and therefore each RG), we compiled all of the core OTUs from the core analysis and removed duplicate bacteria. When doing this, a total of 9, 54, and 16 unique OTUs were assigned to RG1, RG2, and RG3 respectively (**Table 2**). When performing a separate core analysis on each RG as a whole, RG1 was comprised of two core members, a member of the *Winogradskyella* genus (OTU 1511) and a member of the *Bradyrhizobiaceae* family (OTU 6417). While, no core bacterial members were found for RG2 or RG3 microbiomes.

DISCUSSION

The principal goal of this study was to identify patterns in the *C. gigas* microbiome across 35 oyster families with differing levels of resistance to OsHV-1 µvar disease, with the objective of elucidating microbial taxa associated with disease resistance. Immunosuppression from OsHV-1 µvar infection allows opportunistic bacteria within the oyster's microbiome to induce bacteremia, killing the host (Petton et al., 2015; de Lorgeril et al., 2018). Characterizing these interactions and gaining insights into the oyster microbiome is essential to further understand the dynamic interplay between the microbiome,

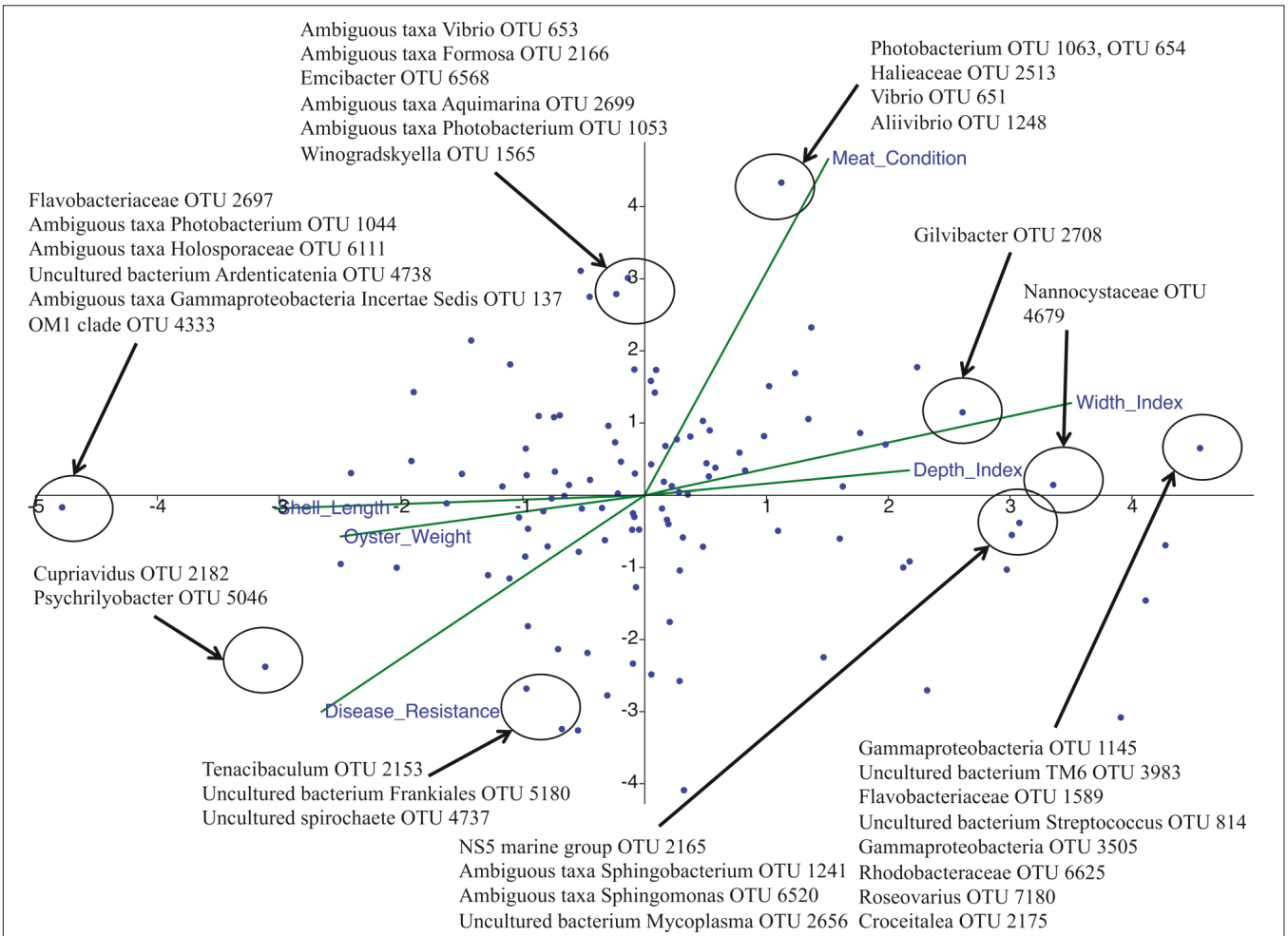


FIGURE 3 | Canonical correspondence analysis (CCA) plot using 3% relative abundance filtered data. *Cupriavidus*, *Psychrilyobacter*, *Tenacibaculum,* and *Frankiales* were found to be strongly associated with OsHV-1 μvar disease-resistance, while OTUs assigned to the *Photobacterium*, *Vibrio,* and *Aliivibrio* were negatively associated with OsHV-1 μvar disease-resistance. Axis 1 and 2 were able to significantly represent 53.2% of the data ($p = 0.001$ for both axes with 999 permutations).

OsHV-1 μvar and disease. A significant difference in the structure of the microbiome of oysters exhibiting different levels of resistance to OsHV-1 μvar disease was observed. Specifically, the microbiomes associated with the oysters showing the most resistance to OsHV-1 μvar disease (RG1) and moderately resistant oysters (RG2) were significantly different to the most disease susceptible (or least resistant) group (RG3). When considering disease resistance, we observed a strong negative association between the OsHV-1 μvar disease resistance of oyster hosts and the occurrence of OTUs assigned to the *Vibrio* (OTUs 651 and 653), *Photobacterium* (OTUs 1063, 654, and 1053), *Aliivibrio* (OTU 1248), *Streptococcus* (OTU 814) and *Roseovarius* (OTU 7180) genera, while on the other hand, the microbiomes of the most resistant families had an over-representation of OTUs assigned to the *Cupriavidus* (OTU 2182), *Psychrilyobacter* (OTU 5046), and *Tenacibaculum* (OTU 2153) genera.

The association between the occurrence of *Vibrio* and disease susceptibility was further supported by a significant elevation of an uncharacterized member of the *Vibrio* in RG3, and the results of a *Vibrio*-specific qPCR assay. These results are consistent with growing evidence implicating a role of the *Vibrio* community in oyster disease (Sugumar et al., 1998; Waechter et al., 2002; Garnier et al., 2007; Saulnier et al., 2010; Lemire et al., 2015; Petton et al., 2015; Green et al., 2018; King et al., 2018). Specifically, there is previous evidence that prior to oyster disease onset, the native *Vibrio* community is replaced by pathogenic *Vibrio* species (Lemire et al., 2015). Further, in corals, small shifts in the *Vibrio* community are sufficient to shift the microbiome metabolism (Thurber et al., 2009). Our data provides a new perspective on this interaction, whereby the total load of *Vibrio* differed between disease susceptible and resistant oyster families. This is supported by a recent study, which demonstrated that the *Vibrio* load following OsHV-1 μvar infection was significantly higher in disease-susceptible oysters (de Lorgeril et al., 2018). An increased *Vibrio* community size may provide further potential for pathogenic species to replace benign colonizers. On the other hand, a higher background load of *Vibrio* may become important under periods of stress, such

TABLE 2 | Unique core bacterial members from individual oyster families collated into their respective resistance groups (RG).

Resistance group	Combined unique core members from individual family lines	
RG1	Acinetobacter OTU_2667	Rhodobacteraceae OTU_6650
	Ambiguous taxa Cellulophaga OTU_3456	Ambiguous taxa Marinomonas OTU_1295
	Brevundimonas OTU_6676	Roseobacter OTU_6715
	Ambiguous taxa Marinomonas OTU_1295	Rhodobacteraceae OTU_7212
	Ambiguous taxa Ilumatobacter OTU_4817	
RG2	Uncultured Salinimonas OTU_6618	Sva0725 OTU_433
	Planctomycetaceae OTU_4123	Uncultured bacterium OM1 clade OTU_4332
	Ambiguous taxa Gammaproteobacteria Incertae Sedis OTU_465	Uncultured bacterium Sva0996 marine group OTU_4477
	Uncultured bacterium Anaerolineaceae OTU_4681	Ambiguous taxa Ilumatobacter OTU_4840
	Uncultured bacteria Gammaproteobacteria OTU_5902	Ambiguous taxa Sva0996 marine group OTU_4982
	Croceitalea OTU_2175	Uncultured bacterium Ardenticatenia OTU_5150
	Uncultured bacterium Halanaerobiales ODP1230B8.23 OTU_4816	Rhodobiaceae OTU_6148
	Oceanospirillaceae OTU_1577	Rhodobacteraceae OTU_6485
	Pseudoalteromonadaceae OTU_3257	Sphingomonadales OTU_6620
	Uncultured bacterium Ilumatobacter OTU_4687	Marivita OTU_6626
	Uncultured bacterium Acidobacteria Subgroup 21 OTU_5711	Beijerinckiaceae OTU_6687
	Rhodobacteraceae OTU_6481	PAUC43f marine benthic group OTU_699
	Ambiguous taxa Acidobacteria Subgroup 9 OTU_7	Anderseniella OTU_7187
	Persicirhabdus OTU_866	Ambiguous taxa Sandaracinaceae OTU_1154
	Uncultured bacterium Ralstonia OTU_1255	Ambiguous taxa Thiogranum OTU_1467
	Flavobacteriaceae OTU_1551	Ambiguous taxa Holophagae Subgroup 23 OTU_251
	Ambiguous taxa Profundimonas OTU_1559	Candidatus Thiobios OTU_6098
	Ambiguous taxa JTB255 marine benthic group OTU_1588	JTB255 marine benthic group OTU_1566
	JTB255 marine benthic group OTU_1642	OM190 OTU_2018
	Gilvibacter OTU_2167	Uncultured bacterium Holophagae Subgroup 23 OTU_240
	Myxococcales OTU_238	Uncultured bacterium Belgica2005-10-ZG-3 OTU_25
	Ambiguous taxa Flavobacteriaceae OTU_3100	Ambiguous taxa Roseibacillus OTU_2529
	Uncultured bacterium Pir4 lineage OTU_3294	OM60 (NOR5) clade OTU_3504
	Halieaceae OTU_3519	Uncultured bacterium Desulfobulbus OTU_404
	Uncultured bacterium OM1 clade OTU_4096	Ambiguous taxa Sva0996 marine group OTU_4976
	Planctomycetaceae OTU_4100	Ambiguous taxa Acidobacteria Subgroup 17 OTU_785
	Ambiguous taxa OM1 clade OTU_4271	
	Planctomycetaceae OTU_4278	
RG3	Rhizobiales OTU_6486	Desulfobulbus OTU_235
	Uncultured bacterium Rickettsiaceae OTU_5903	Uncultured bacterium Emcibacter OTU_6286
	Vibrionaceae OTU_655	Rhodobacteraceae OTU_7097
	Ambiguous taxa Sphingobacterium OTU_1241	Mycoplasma OTU_3722
	Ambiguous taxa NS4 marine group OTU_2698	Uncultured bacterium Phyllobacteriaceae OTU_6619
	Pseudomonas OTU_3032	Ruegeria OTU_6653
	Gammaproteobacteria OTU_3505	Uncultured bacterium Rhodobacteraceae OTU_7173
	Roseovarius OTU_7180	
	Uncultured bacterium Maribacter OTU_1486	

(Continued)

TABLE 2 | Continued

Each family was found to have a core microbial community, the displayed core OTUs are those not shared with any other family line. RG1 is the most disease RG, RG2 is an intermediate RG, and RG3 is the most disease susceptible group.

as with OsHV-1 μvar infection, resulting in duel infection, as has recently been described (de Lorgeril et al., 2018). This is also indirectly supported by a previous study which observed reduced mortality in OsHV-1 infected oysters that were treated with antibiotics (Petton et al., 2015).

Increases in the abundance of OTUs assigned to the *Photobacterium* genus, as were observed here, often co-occur with an increase in the *Vibrio* community in oyster microbiomes (Wegner et al., 2013; Lokmer and Wegner, 2015). While members assigned to this genus have been identified as pathogens of other aquatic organisms (Pedersen et al., 2009; Liu et al., 2016), to our knowledge, no species of *Photobacterium* has been identified as an oyster pathogen. Members of the *Streptococcus* and *Aliivibrio* genera are known pathogens of fish and crabs (Pappalardo and Boemare, 1982; Egidius et al., 1986; Creeper and Buller, 2006; Urbanczyk et al., 2007), while a member of the *Roseovarius* genus is the causative agent of roseovarius oyster disease (formally juvenile oyster disease) in *Crassostrea virginica* (Boettcher et al., 2005; Maloy et al., 2007), yet to our knowledge these genera have not been implicated in disease of *C. gigas* previously, despite being over-represented in the most disease susceptible oyster families.

On the other hand, a strong positive association was observed between levels of disease resistance and the occurrence of OTUs assigned to the *Cupriavidus* (OTU 2182), *Psychrilyobacter* (OTU 5046) and *Tenacibaculum* (OTU 2153). Currently, little is known about the role of these genera in oysters. *Cupriavidus* species are commonly isolated from plants and soil (Cuadrado et al., 2010; Estrada-De Los Santos et al., 2014), but members of the *Psychrilyobacter* and *Tenacibaculum* have previously been observed in *C. gigas* microbiomes (Lee et al., 2009; Fernandez-Piquer et al., 2012; Wegner et al., 2013). *Psychrilyobacter* was observed in *C. gigas* microbiomes from Tasmania, Australia (Fernandez-Piquer et al., 2012), which is perhaps notable given that the oysters used in this study were initially sourced from Tasmania. In addition, we have previously identified an over-representation of a *Tenacibaculum* OTU in oyster microbiomes that were unaffected by a summer mortality outbreak (King et al., 2018).

As already stated, a significant elevation of OTUs belonging to the *Vibrio* and *Photobacterium* genera abundance in disease susceptible oysters has also been previously observed (de Lorgeril et al., 2018), supporting our findings. However, while we identified members of the *Psychrilyobacter* and *Tenacibaculum* genera to be associated with disease resistance, the same study (de Lorgeril et al., 2018) observed an increase in these same genera in an experimental infection experiment using disease susceptible oysters. Differences in bacterial taxa abundance and taxonomic assignment could be attributed to contrasting sequencing techniques and data analysis. For example, we used the V1–V3 hypervariable region, and clustered OTUs at the 97% identity level, compared to V3–V4 and having OTUs clustered at a three-nucleotide difference threshold (de Lorgeril et al., 2018). Furthermore, this study deployed oysters to the field, while the aforementioned study carried out their experiments in tanks. Tank based studies are known to significantly alter the oyster microbiome composition compared to oysters sourced from the environment (Lokmer et al., 2016a).

The oyster microbiome is dynamic in nature, changing in response to stressors such as disease, antibiotics, translocation, and heat (Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016b; de Lorgeril et al., 2018; Green et al., 2018; King et al., 2018). The microbiome assemblage can also be influenced by the oyster life stage, the genetics of the host oyster, and spatial location (Trabal et al., 2012; Wegner et al., 2013; Lokmer et al., 2016a; King et al., 2018). Because we only have one sampling point, our study would not capture the dynamic nature of the oyster microbiome, and thus the oyster microbiome could change before the onset of disease. To fully capture the importance of the taxa identified in this study, a temporal study in the field encompassing a disease outbreak would be needed. However, as disease outbreaks are often very sudden, capturing a disease outbreak in the environment can be difficult.

In addition to identifying OTUs that are over- or under-represented within the microbiomes of oysters with different levels of disease-resistance, another way to identify putatively important bacteria within the microbiome of a host organism involves the identification of "core" microbiome members (Ainsworth et al., 2015). Identifying which bacterial members are consistent and stable across microbial communities is important in unraveling the functional contribution of these core bacteria (Ainsworth et al., 2015). Notably, we could not define a universal core microbiome across all of the studied oyster families at the OTU level, suggesting significant heterogeneity in oyster microbiome structure, or possible differences in micro-geographic variation. However, we identified core microbiome members within each family microbiome, whereby a number of 'unique' core members often occurred exclusively in the core microbiome of a family. This is in accordance with previous observations that the composition of an oyster's microbiome is partially governed by oyster genetics, particularly for shaping the rare specialist bacterial community (<1% abundance) (Wegner et al., 2013), although we have no information pertaining to the genetic differentiation between the studied oyster families. However, when examining the core microbiome across all of the families comprising the most highly disease-resistant group (RG1), we identified two core members, which included OTUs classified as members of the *Winogradskyella* genus (OTU 1511) and *Bradyrhizobiaceae* family (OTU 6417). OTUs assigned to the *Bradyrhizobiaceae* family have previously been observed in oysters (Sakowski, 2015), however, due to the coarse taxonomic assignment of this OTU, it is unclear what potential role this member of the *Bradyrhizobiaceae* family might have. *Winogradskyella* species are commonly found in numerous marine organisms, including oysters (Valdenegro-Vega et al., 2013; Park et al., 2015; Lee et al., 2017; Schellenberg et al., 2017; Franco et al., 2018), and are known for their role in amoebic-induced fish gill diseases (Embar-Gopinath et al., 2005, 2006). However, it is uncertain what function(s) *Winogradskyella* species play in oysters. We currently know little about the potential role, if any, of these core microbiome members in resistance, but these observations provide candidate target organisms for focused examinations of potential beneficial microbes within OsHV-1 μvar disease-resistance.

CONCLUSION

We have shown that the microbiome of *C. gigas* displays significantly different microbial assemblage structure according to oyster disease-resistance. This study provides insights into the *C. gigas* microbiome within the context of oysters bred for disease-resistance and highlights the potential involvement of the oyster microbiome in disease-resistance. Members of the *Vibrio*, *Photobacterium*, *Aliivibrio*, *Streptococcus,* and *Roseovarius* genera were over-represented features of the microbiome of oysters with high OsHV-1 μvar disease susceptibility, which is consistent with previous studies implicating *Vibrio* in oyster disease dynamics. Furthermore, a significant elevation of *Vibrio* 16S rRNA gene copies in disease-susceptible oyster families could indicate a lack of immune response against *Vibrio* pathogens. However, further research is required to elucidate the role of these bacteria in oyster disease dynamics. Examination of 'core' bacteria identified species assigned to the *Winogradskyella* genus and *Bradyrhizobiaceae* family as core members of microbiomes assigned to RG1 and may also play a role in OsHV-1 μvar disease resistance. These results deliver evidence that the *C. gigas* microbiome differs between oysters with different levels of susceptibility to OsHV-1 μvar disease and identifies putative microbial determinants in disease onset and resistance.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI SRA, PRJNA497763.

AUTHOR CONTRIBUTIONS

WK and KN carried out the fieldwork. WK and NW processed the samples. WK and NS analyzed the data. CJ, MD, WO'C,

JS, and ML conceived and designed the study. TK produced the core microbiome analysis. WK, JS, and ML wrote the manuscript.

FUNDING

This research was supported by an Australian Research Council Linkage Project (LP160101795) to JS and ML; 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.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00473/full#supplementary-material

REFERENCES

Ainsworth, T. D., Krause, L., Bridge, T., Torda, G., Raina, J.-B., Zakrzewski, M., et al. (2015). The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *ISME J.* 9, 2261–2275. doi: 10.1038/ismej.2015.39

ASI (2015). *Australian Seafood Industries Oyster Breeding Programs*. Available at: http://www.asioysters.com.au/breeding-program.html

Azéma, P., Travers, M. A., De Lorgeril, J., Tourbiez, D., and Dégremont, L. (2015). Can selection for resistance to OsHV-1 infection modify susceptibility to *Vibrio aestuarianus* infection in *Crassostrea gigas*? First insights from experimental challenges using primary and successive exposures. *Vet. Res.* 46:139. doi: 10.1186/s13567-015-0282-0

Boettcher, K. J., Geaghan, K. K., Maloy, A. P., and Barber, B. J. (2005). *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter clade* and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *Int. J. Syst. Evol. Microbiol.* 55, 1531–1537. doi: 10.1099/ijs.0.63620-0

Burge, C. A., Griffin, F. J., and Friedman, C. S. (2006). Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. *Dis. Aquat. Organ.* 72, 31–43. doi: 10.3354/dao072031

Camara, M. D., Yen, S., Kaspar, H. F., Kesarcodi-Watson, A., King, N., Jeffs, A. G., et al. (2017). Assessment of heat shock and laboratory virus challenges to selectively breed for ostreid herpesvirus 1 (OsHV-1) resistance in the Pacific oyster, *Crassostrea gigas*. *Aquaculture* 469, 50–58. doi: 10.1016/j.aquaculture.2016.11.031

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Creeper, J. H., and Buller, N. B. (2006). An outbreak of *Streptococcus iniae* in barramundi (*Lates calcarifera*) in freshwater cage culture. *Aust. Vet. J.* 84, 408–411. doi: 10.1111/j.1751-0813.2006.00058.x

Cuadrado, V., Gomila, M., Merini, L., Giulietti, A. M., and Moore, E. R. B. (2010). *Cupriavidus pampae* sp. nov., a novel herbicide-degrading bacterium isolated from agricultural soil. *Int. J. Syst. Evol. Microbiol.* 60, 2606–2612. doi: 10.1099/ijs.0.018341-0

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., et al. (2005). A novel class of herpesvirus with bivalve hosts. *J. Gen. Virol.* 86, 41–53. doi: 10.1099/vir.0.80382-0

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

Dégremont, L. (2011). Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. *Aquaculture* 317, 94–98. doi: 10.1016/j.aquaculture.2011.04.029

Dégremont, L. (2013). Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. *Aquaculture* 416, 129–134. doi: 10.1016/j.aquaculture.2013.09.011

Dégremont, L., Lamy, J. B., Pépin, J. F., Travers, M. A., and Renault, T. (2015a). New insight for the genetic evaluation of resistance to ostreid herpesvirus infection, a worldwide disease, in *Crassostrea gigas*. *PLoS One* 10:e0127917. doi: 10.1371/journal.pone.0127917

Dégremont, L., Nourry, M., and Maurouard, E. (2015b). Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: response to selection after four generations. *Aquaculture* 446, 111–121. doi: 10.1016/j.aquaculture.2015.04.029

Dégremont, L., Ledu, C., Maurouard, E., Nourry, M., and Benabdelmouna, A. (2016a). Effect of ploidy on the mortality of *Crassostrea gigas* spat caused by OsHV-1 in France using unselected and selected OsHV-1 resistant oysters. *Aquac. Res.* 47, 777–786. doi: 10.1111/are.12536

Dégremont, L., Morga, B., Trancart, S., and Pépin, J. F. (2016b). Resistance to OsHV-1 infection in *crassostrea gigas* larvae. *Front. Mar. Sci.* 3:15. doi: 10.3389/fmars.2016.00015

Dégremont, L., Tanguy, G., Delphine, T., and Jean-François, P. (2013). Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles? *Aquaculture* 408–409, 51–57. doi: 10.1016/j.aquaculture.2013.05.025

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

Egidius, E., Wiik, R., Andersen, K., Hoff, K. A., and Hjeltnes, B. (1986). *Vibrio salmonicida* sp. nov., a new fish pathogen. *Int. J. Syst. Evol. Microbiol.* 36, 518–520. doi: 10.1099/00207713-36-4-518

Embar-Gopinath, S., Butler, R., and Nowak, B. (2005). Influence of salmonid gill bacteria on development and severity of amoebic gill disease. *Dis. Aquat. Organ.* 67, 55–60. doi: 10.3354/dao067055

Embar-Gopinath, S., Crosbie, P., and Nowak, B. F. (2006). Concentration effects of *Winogradskyella* sp. on the incidence and severity of amoebic gill disease. *Dis. Aquat. Organ.* 73, 43–47. doi: 10.3354/dao073043

Estrada-De Los Santos, P., Solano-Rodríguez, R., Matsumura-Paz, L. T., Vásquez-Murrieta, M. S., and Martínez-Aguilar, L. (2014). *Cupriavidus plantarum* sp. nov., a plant-associated species. *Arch. Microbiol.* 196, 811–817. doi: 10.1007/s00203-014-1018-7

Fernandez-Piquer, J., Bowman, J. P., Ross, T., and Tamplin, M. L. (2012). Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure. *J. Appl. Microbiol.* 112, 1134–1143. doi: 10.1111/j.1365-2672.2012.05287.x

Franco, A., Busse, H. J., Schubert, P., Wilke, T., Kämpfer, P., and Glaeser, S. P. (2018). *Winogradskyella pocilloporae* sp. nov, isolated from healthy tissue of the coral *Pocillopora damicornis*. *Int. J. Syst. Evol. Microbiol.* 68, 1689–1696. doi: 10.1099/ijsem.0.002731

Friedman, C. S., Estes, R. M., Stokes, N. A., Burge, C. A., Hargove, J. S., Barber, B. J., et al. (2005). Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. *Dis. Aquat. Organ.* 63, 33–41. doi: 10.3354/dao063033

Garcia, C., Thebault, A., Degremont, L., Arzul, I., Miossec, L., Robert, M., et al. (2011). Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. *Vet. Res.* 42:73. doi: 10.1186/1297-9716-42-73

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

Go, J., Deutscher, A., Spiers, Z., Dahle, K., Kirkland, P., and Jenkins, C. (2017). An investigation into mass mortalities of unknown aetiology in Pacific oysters,

Crassostrea gigas, in Port Stephens, New South Wales, Australia. *Dis. Aquat. Organ.* 125, 227–242. doi: 10.3354/dao03146

Green, T. J., Siboni, N., King, W. L., Labbate, M., Seymour, J. R., and Raftos, D. (2018). Simulated marine heat wave alters abundance and structure of *Vibrio* populations associated with the Pacific Oyster resulting in a mass mortality event. *Microb. Ecol.* doi: 10.1007/s00248-018-1242-9 [Epub ahead of print].

Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2001). Past: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* 4, 1–9.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S. A., Gu, X., et al. (2013). Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in *Crassostrea gigas* (Pacific oysters) in Australia. *Dis. Aquat. Organ.* 105, 109–126. doi: 10.3354/dao02623

Kahlke, T. (2017). *Panbiom*. Available at: https://github.com/timkahlke/panbiom

Keeling, S. E., Brosnahan, C. L., Williams, R., Gias, E., Hannah, M., Bueno, R., et al. (2014). New Zealand juvenile oyster mortality associated with ostreid herpesvirus 1-an opportunistic longitudinal study. *Dis. Aquat. Organ.* 109, 231–239. doi: 10.3354/dao02735

King, W. L., Jenkins, C., Go, J., Siboni, N., Seymour, J. R., and Labbate, M. (2018). Characterisation of the Pacific Oyster microbiome during a summer mortality event. *Microb. Ecol.* doi: 10.1007/s00248-018-1226-9 [Epub ahead of print].

King, W. L., Jenkins, C., Seymour, J. R., and Labbate, M. (2019). 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

Kube, P., Dove, M., Cunningham, M., Kirkland, P., Gu, X., Hick, P., et al. (2018). *Genetic Selection for Resistance to Pacific Oyster Mortality Syndrome*. Available: http://www.asioysters.com.au/uploads/2/5/9/7/25976125/kube_et_al__2018__genetic_selection_for_resistance_to_poms_-_sfcrc_project_final_report.pdf [accessed December 15, 2018].

Lee, J. H., Kang, J. W., Shin, S. B., and Seong, C. N. (2017). *Winogradskyella flava* sp. nov., isolated from the brown alga, *Sargassum fulvellum*. *Int. J. Syst. Evol. Microbiol.* 67, 3540–3546. doi: 10.1099/ijsem.0.002161

Lee, Y. S., Baik, K. S., Park, S. Y., Kim, E. M., Lee, D. H., Kahng, H. Y., et al. (2009). *Tenacibaculum crassostreae* sp. nov., isolated from the Pacific oyster, *Crassostrea gigas*. *Int. J. Syst. Evol. Microbiol.* 59, 1609–1614. doi: 10.1099/ijs.0.006866-0

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

Liu, F., Liu, G., and Li, F. (2016). Characterization of two pathogenic *Photobacterium* strains isolated from *Exopalaemon carinicauda* causing mortality of shrimp. *Aquaculture* 464, 129–135. doi: 10.1016/j.aquaculture.2016.06.019

Lokmer, A., Goedknegt, M. A., Thieltges, D. W., Fiorentino, D., Kuenzel, S., Baines, J. F., et al. (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., and Wegner, K. M. (2016b). The role of tissue-specific microbiota in initial establishment success of Pacific oysters. *Environ. Microbiol.* 18, 970–987. doi: 10.1111/1462-2920.13163

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

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

Maloy, A. P., Ford, S. E., Karney, R. C., and Boettcher, K. J. (2007). *Roseovarius crassostreae*, the etiological agent of Juvenile Oyster Disease (now to be known as Roseovarius Oyster Disease) in *Crassostrea virginica*. *Aquaculture* 269, 71–83. doi: 10.1016/j.aquaculture.2007.04.008

Moreau, P., Moreau, K., Segarra, A., Tourbiez, D., Travers, M. A., Rubinsztein, D. C., et al. (2015). Autophagy plays an important role in protecting Pacific oysters from OsHV-1 and *Vibrio aestuarianus* infections. *Autophagy* 11, 516–526. doi: 10.1080/15548627.2015.1017188

Mortensen, S., Strand, A., Bodvin, T., Alfjorden, A., Skar, C. K., Jelmert, A., et al. (2016). Summer mortalities and detection of ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. *Dis. Aquat. Organ.* 117, 171–176. doi: 10.3354/dao02944

Neuwirth, E. (2014). *RColorBrewer: ColorBrewer Palettes*. Available at: https://cran.r-project.org/web/packages/RColorBrewer/index.html

Pappalardo, R., and Boemare, N. (1982). An intracellular *Streptococcus*, causative agent of a slowly developing disease in the Mediterranean crab, *Carcinus mediterraneus*. *Aquaculture* 28, 283–292. doi: 10.1016/0044-8486(82)90070-9

Park, S., Park, J. M., Won, S. M., and Yoon, J. H. (2015). *Winogradskyella crassostreae* sp. nov., isolated from an oyster (*Crassostrea gigas*). *Int. J. Syst. Evol. Microbiol.* 65, 2890–2895. doi: 10.1099/ijs.0.000350

Paul-Pont, I., Dhand, N. K., and Whittington, R. J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. *Aquaculture* 412-413, 202–214. doi: 10.1016/j.aquaculture.2013.07.038

Pedersen, K., Skall, H. F., Lassen-Nielsen, A. M., Bjerrum, L., and Olesen, N. J. (2009). *Photobacterium damselae* subsp. *damselae*, an emerging pathogen in Danish rainbow trout, *Oncorhynchus mykiss* (Walbaum), mariculture. *J. Fish Dis.* 32, 465–472. doi: 10.1111/j.1365-2761.2009.01041.x

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., and 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

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reshef, D. N., Reshef, Y. A., Finucane, H. K., Grossman, S. R., Mcvean, G., Turnbaugh, P. J., et al. (2011). Detecting novel associations in large data sets. *Science* 334, 1518–1524. doi: 10.1126/science.1205438

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

Sakowski, E. G. (2015). *The Microbiome of the Eastern Oyster, Crassostrea virginica, in Health and Disease*. Ph.D. thesis, University of Delaware, Newark, DE.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., and 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, 787–798. doi: 10.1007/s00248-009-9620-y

Sauvage, C., Pépin, J. F., Lapègue, S., Boudry, P., and Renault, T. (2009). Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR. *Virus Res.* 142, 181–187. doi: 10.1016/j.virusres.2009.02.013

Schellenberg, J., Busse, H. J., Hardt, M., Schubert, P., Wilke, T., Kämpfer, P., et al. (2017). *Winogradskyella haliclonae* sp. Nov., isolated from a marine sponge of the genus Haliclona. *Int. J. Syst. Evol. Microbiol.* 67, 4902–4910. doi: 10.1099/ijsem.0.002192

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

Segarra, A., Mauduit, F., Faury, N., Trancart, S., Dégremont, L., Tourbiez, D., et al. (2014). Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. *BMC Genomics* 15:580. doi: 10.1186/1471-2164-15-580

Segarra, A., Pepin, J. F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* 153, 92–99. doi: 10.1016/j.virusres.2010.07.011

Shin, S. C., Kim, S. H., You, H., Kim, B., Kim, A. C., Lee, K. A., et al. (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* 334, 670–674. doi: 10.1126/science.1212782

Shreiner, A. B., Kao, J. Y., and Young, V. B. (2015). The gut microbiome in health and in disease. *Curr. Opin. Gastroenterol.* 31, 69–75. doi: 10.1097/MOG.0000000000000139

Siboni, N., Balaraju, V., Carney, R., Labbate, M., and Seymour, J. R. (2016). Spatiotemporal dynamics of *Vibrio* spp. within the Sydney Harbour estuary. *Front. Microbiol.* 7:460. doi: 10.3389/fmicb.2016.00460

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., and Muroga, K. (1998). *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster

Crassostrea gigas larvae. *Dis. Aquat. Organ.*33, 111–118. doi: 10.3354/dao033111

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E., and Polz, M. F. (2004). Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Appl. Environ. Microbiol.* 70, 4103–4110. doi: 10.1128/AEM.70.7.4103-4110.2004

Thurber, R. V., Willner-Hall, D., Rodriguez-Mueller, B., Desnues, C., Edwards, R. A., Angly, F., et al. (2009). Metagenomic analysis of stressed coral holobionts. *Environ. Microbiol.* 11, 2148–2163. doi: 10.1111/j.1462-2920.2009.01935.x

Trabal, N., Mazon-Suastegui, J. M., Vazquez-Juarez, R., Asencio-Valle, F., Morales-Bojorquez, E., and 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, 555–569. doi: 10.1007/s00248-012-0039-5

Urbanczyk, H., Ast, J. C., Higgins, M. J., Carson, J., and Dunlap, P. V. (2007). Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int. J. Syst. Evol. Microbiol.* 57, 2823–2829. doi: 10.1099/ijs.0.65081-0

Valdenegro-Vega, V., Naeem, S., Carson, J., Bowman, J. P., Tejedor Del Real, J. L., and Nowak, B. (2013). Culturable microbiota of ranched southern bluefin tuna (*Thunnus maccoyii* Castelnau). *J. Appl. Microbiol.* 115, 923–932. doi: 10.1111/jam.12286

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P. C., Colwell, R. R., Höfle, M. G., et al. (2011). Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. *ISME J.* 6, 21–30. doi: 10.1038/ismej.2011.89

Waechter, M., Le Roux, F., Nicolas, J. L., Marissal, E., and Berthe, F. (2002). Characterisation of *Crassostrea gigas* spat pathogenic bacteria. *C. R. Biol.* 325, 231–238. doi: 10.1016/S1631-0691(02)01428-2

Warnes, G. R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W. H. A., Lumley, T., et al. (2016). *gplots: Various R Programming Tools for Plotting Data. R Package Version 3.0.1*. Available at: https://mran.microsoft.com/snapshot/2017-04-11/web/packages/gplots/index.html

Wegner, K. M., Volkenborn, N., Peter, H., and Eiler, A. (2013). Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiol.* 13:252. doi: 10.1186/1471-2180-13-252

Whittington, R. J., Dhand, N. K., Evans, O., and Paul-Pont, I. (2015). Further observations on the influence of husbandry practices on OsHV-1 μVar mortality in Pacific oysters *Crassostrea gigas*: age, cultivation structures and growing height. *Aquaculture* 438, 82–97. doi: 10.1016/j.aquaculture.2014.12.040

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.