CHARACTERIZING THE MICROBIOME OF FARMED PACIFIC OYSTERS FROM BRITISH COLUMBIA AND ALASKA

by

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Characterizing the microbiome of farmed Pacific oysters from British Columbia and Alaska

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Abstract

Pacific oyster (*Crassostrea gigas*) aquaculture is one of the largest suppliers of seafood worldwide\(^1\). For instance, oyster production from British Columbia contributes 60% of the annual shellfish production in Canada. With the expanding industry, there have been growing incidents of mortality events in hatchery production, causing the seed stock shortages limiting sales to nursery growers\(^2\)–\(^4\). Recent studies have attempted to characterize the microbiome (pathobiome) associated with mortality; however, differing effects of harvest sites, tissues, age, and abiotic stresses on the oyster microbiome make it challenging to identify the mortality causes\(^5\)–\(^9\). Thus, there is a need to define the core members of oyster microbiome to serve as a baseline for future hypotheses testing. In the current study, Pacific oysters were obtained from two farm sites differing in location, sampling time, and production stages with the goal of identifying microbial taxa that are consistently found in *C. gigas*. Bacteria in the genus *Sulfitobacter* and phylum *Planctomycetes*, which are known to oxidize sulfur and fix nitrogen, respectively\(^10\)–\(^13\), were identified in all samples. To determine taxa only associated with mortality events, the microbiome of post-metamorphosis hatchery oysters (spat) that had experienced mortality were compared to spat that had not. Bacteria in the order *Alteromonadales* and genus *Roseovarius* were associated with spat sampled before, during and after peak mortality. No significant (Kruskal Wallis pair-wise; \(p>0.05\)) differences in microbiome composition and diversity were observed between populations that experienced or did not experience mortality. Irrespective of mortality events, however, the composition and relative abundance of microbial taxa changed as the oysters aged, with the highest dissimilarity in microbial composition and diversity occurring among the youngest spat. High dissimilarity in the microbiome of the youngest spat may reflect; i) diseased states of developing spat, some of which experienced
mortality while others showed a stochastic microbiome pattern and, ii) a “maturing” microbiome after metamorphosis in rapidly developing spat\textsuperscript{5,6,14}. The common taxa identified in the oyster samples examined in this study are linked to important biogeochemical cycles and may be part of a core microbiome related to the filter-feeding behaviour of oysters and the surrounding environment.
Lay Summary

Pacific oyster is the most widely cultivated oyster in the shellfish aquaculture worldwide. In Canada, Pacific oysters are only farmed in British Columbia, where they comprised an average of 60% of Canada’s oyster production value from 2011-2015. Despite its contribution in meeting global seafood demand, hatcheries are experiencing frequent mass-mortality events resulting in shortages of seed stock. To investigate potential causes of the mortality events, microbial communities (microbiome) structure within the seed stocks were examined and compared between mortality and healthy groups. Although no significant difference in microbiome structure was observed between the two groups, rapid shifts in the microbiome composition occurred as the oyster spat developed. The finding implies that the mortality events were linked to stabilization and integrity of rapidly shifting microbiome during the early development, alerting greater need to focus on characterizing the interaction between the early development and microbiome.
Preface

I performed all the laboratory work pertinent to the thesis, including sample processing, DNA extraction (except six samples which were extracted by Dr Jing-Zhe Jiang), polymerase chain reaction, and sequencing library preparations. The library preparation protocol, 16S RNA primer selection, and training were provided by a post-doctoral fellow Dr Xu Kevin Zhong (unpublished work). I analyzed all the amplicon sequence data and produced figures using QIIME2, RStudio, and Unix. Dr Jan Finke (post-doctoral fellow) helped with various statistical analyses during the data analysis. Dr Curtis Suttle, Amy Chan (research scientist) and I selected appropriate oyster samples for the study based on mortality events.

All the BC oyster samples were from Island Scallops Ltd. and were provided by Rob Saunders in 2014; they were stored at Pacific Biological Station (Department of Fisheries and Oceans, Nanaimo, BC) until processed for this study. All the AK oyster and filtered water samples were provided by Kachemak Shellfish Grower’s Association, who followed sampling protocols and kits provided by Amy Chan (Research Scientist in the lab) and me.

Chapter 2 is part of an ongoing collaborative study on Sustainable Oyster Aquaculture in British Columbia.
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<td>Alaska</td>
</tr>
<tr>
<td>AKP</td>
<td>Anna Karenina Principle</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ANOSIM</td>
<td>Analysis of Similarities</td>
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<tr>
<td>AM</td>
<td>After Mortality peak</td>
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<tr>
<td>BC</td>
<td>British Columbia</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BM</td>
<td>Before Mortality peak</td>
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<tr>
<td>CCA</td>
<td>Canonical Correlation Analysis</td>
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<tr>
<td>DADA2</td>
<td>Divisive Amplicon Denoising Algorithm 2</td>
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<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
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<tr>
<td>DM</td>
<td>During Mortality peak</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA pol</td>
<td>DNA polymerase</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
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<tr>
<td>dsDNA</td>
<td>Double-strand DNA</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
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<tr>
<td>FLUPSY</td>
<td>Floating Upweller System</td>
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<tr>
<td>IndVal</td>
<td>Indicator Value Index</td>
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<tr>
<td>ISL</td>
<td>Island Scallops Ltd.</td>
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<tr>
<td>KSG</td>
<td>Kachemak Shellfish Growers</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LCBD</td>
<td>Local Contribution to Beta Diversity</td>
</tr>
<tr>
<td>MAFFT</td>
<td>Multiple Alignment using Fast Fourier Transform</td>
</tr>
<tr>
<td>MSX</td>
<td>Multi-nucleated unknown X</td>
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<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
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<tr>
<td>nMDS</td>
<td>Nonmetric Multi-Dimensional Scaling</td>
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<tr>
<td>OsHV</td>
<td>Osterid Herpes Virus</td>
</tr>
<tr>
<td>OTUs</td>
<td>Operational Taxonomic Units</td>
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<tr>
<td>PCoA</td>
<td>Principal Coordinate Analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<tr>
<td>QIIME2</td>
<td>Quantitative Insights Into Microbial Ecology 2</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SSU 16S rRNA</td>
<td>Small Subunit 16S Ribosomal RNA</td>
</tr>
<tr>
<td>SoG</td>
<td>Strait of Georgia</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Terminal Restriction Fragment Length Polymorphism</td>
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<td>UBC</td>
<td>University of British Columbia</td>
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</table>
Glossary

Anna Karenina principle (AKP) A hypothesis predicting that certain stressors have stochastic rather than deterministic effects on host-associated microbiome composition.

Alpha-diversity Biodiversity at a local scale, often characterized by number-of-species counts and how evenly different species comprise the microbiome.

Amplicon A region of genetic sequence amplified by using the polymerase chain reaction.

Beta-diversity Variation of species structure among sites, times, or designated groupings; often measured by relative abundance of read counts or phylogenetic distance.

Bray-Curtis dissimilarity A statistical method to differentiate species composition between two groups based on the abundance of read counts.

Broodstock Mature animals used as a source for seed stock in aquaculture.

Commensal Microbes that are associated with a host that has neutral effect on host survival upon colonization.

Core microbiome Members of a common microbial assemblage associated with defined habitats, environmental variables, hosts or host tissues. In this study, it was defined as operational taxonomic units with prevalent and abundant sequence reads.

D-stage larvae Oyster larvae developmental stage during which the fertilized eggs have the characteristic D-shaped shell valves.
**Depuration** A purification process in which shellfish are placed in sterile water to allow the intestinal contents (including microbes) to be expelled during filtration.

**Dysbiosis** A shift in the microbial community that has negative impact on the host.

**Epibionts** Organisms or microbes that reside on the surface of another organisms (e.g. oyster shell).

**Holobiont** Collective term to refer to a host and associated microbes, under healthy conditions.

**Holobiont theory of evolution** A theory that considers holobiont and the sum of its genetic information (i.e. hologenome) as a unit of selection in evolution.

**Juvenile oysters** Although ‘juveniles’ are most frequently used describing developing oysters from larvae, seeds or spat, the juvenile oysters in this study refer to oysters that are between 2-4 cm that have not reached market size (>6 cm).

**Local Contribution to Beta Diversity (LCBD)** A statistical analysis that measures per-sample contribution to total beta diversity. By looking at LCBD value, one can determine how different the microbial community structure of a single sample is from average beta diversity of all samples.

**Microbiome** A characteristic microbial community occupying a given habitat, often characterized by assemblages of genetic sequences.

**Microbiota** A term interchangeably used with “microbiome” in this study; usually referring to specific groups of microbial communities associated with specific environments, spatio-temporal variables, hosts, or stressors.
**Operational taxonomic unit** A bioinformatic classification unit of processing sequence data based on sequence similarity and relatedness.

**Pathobiome** A concept representing a pathogenic agent integrated within a microbial community which may show characteristic microbiome structure associated with diseased hosts or susceptible state.

**Pielou** A mathematical measure of biodiversity to quantify species evenness (i.e. how equal the number of each species is) in a community

**Richness** A biodiversity measure describing the number of species found in a community, often quantified by counting unique OTUs.

**Seed** A term used in aquaculture to refer to fertilized eggs that are to start a production batch; it is sometimes used interchangeably with the term, “spat”.

**Shannon Index** Shannon-Wiener index; a biodiversity measurement that accounts for species richness and the proportion of each species within a community.

**SILVA** Online reference database providing aligned small- and large-subunit rRNA sequences for all three domains of life.

**Spatting** A process of oyster larvae settling on a hard substrate as part of metamorphosis.

**Spat** Post-metamorphic Pacific oysters, ranging between 1 and 3 mm in size.

**Strip-spawning** A method to artificially fertilize oyster embryos in aquaculture by sacrificing broodstock by stripping off their gonads.
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I would like to thank to all Suttle lab members and visiting scientists for their guidance, feedback, encouragement, warmth, and joy. I would especially like to extend my gratitude to Amy Chan, Anwar Al-Qattan, Jessica Caleta, Tianyi Chang, Christoph Deeg, Jan Finke, Alireza Golnaraghi, Junya Hirai, Ezra Kitson, Andrew Lang, Gideon Mordecai, Jeffrey Strohm, Marli Vlok, Rui Zhang, Xu (Kevin) Zhong, and Matthias Zimmer. Many thanks to Kevin Zhong especially for guiding me through lab work a step at a time patiently.

Additional thanks to Vittorio Boscaro, Amy Chan, Chris Deeg, Jan Finke, Emma George, Angela Schulze, Marli Vlok and Kevin Zhong for helping with trouble-shooting, data analysis, editing, and presentations.

I would also like to thank Rob Saunders for providing the Island Scallops Ltd. oyster samples and advice for the thesis. His passion for science and aquaculture has been inspirational. I would also like to thank Kachemak Growers Association for providing the oysters and water samples despite the busy schedule.
I have received generous funding during my MSc degree program. I have been supported by North Pacific Research Board (NPRB), UBC Ocean Leaders Graduate Fellowship and Metagenomic Data Analysis Workshop Registration Award (Canadian Bioinformatics Workshops). The lab and the project have been supported by Natural Sciences and Engineering Research Council of Canada (NSERC), Moore Foundation, and Aquaculture Collaborative Research and Development Program (ACRDP).

Thanks to all the friends, office mates, and Biodiversity Research Centre floor mates for food, coffee, chocolates, camping, climbing, Friday Beer Social, lunch table laughter, and making the experience worth so much more than a degree.

Finally, I would like to express my deep gratitude and respect for my parents and brother for their continued support and love throughout this degree. I would not have been where I am at without them.
Dedication

For my loving family who never cease to support, have hope, and pray in God.

I would not have been where I am without their guidance and love.

“Be joyful, pray continuously, and give thanks in all circumstances; for this is God’s will for you in Christ Jesus” 1 Thessalonians 5:16-18 NIV

And for penguins.
Chapter 1: Introduction

Characterization of microbial communities can be used to answer ecological, physiological, aetiological and evolutionary questions as microbiomes are found in various ecological niches, including in animal tissues and water. As a community unit, microbes can have a significant role in their host’s health and development by contributing to host metabolic process\textsuperscript{15}, immune response\textsuperscript{16,17}, and homeostatic maintenance\textsuperscript{18,19}. The microbiome is in turn influenced by the environment in which the host resides, food intake, medication, physiological changes, and diseases\textsuperscript{6,20–22}. The integrity of the microbiome is assessed by the species composition, richness, diversity\textsuperscript{23}, and evenness\textsuperscript{24}, all of which can be used as indices for gauging the host’s health in relation to various biotic and abiotic factors\textsuperscript{23–26}. Consequently, microbiomes are a useful tool for understanding host-microbe, host-environment, and host-host interactions, as has been demonstrated in medicine\textsuperscript{27}, microbial ecology\textsuperscript{28}, and agriculture\textsuperscript{21,29,30}.

One of the growing applications of studying microbiomes is to investigate potential causes of diseases in marine animals including aquaculture. Compared to terrestrial livestock, the extent of environmental contact and its influence on marine animals may be larger due to the frequent co-dispersion of feed and waste in the surrounding water\textsuperscript{26}. As a result, slight changes in water quality (e.g. temperature, pH, or emerging pathogens) can induce shifts in the composition of aquatic microbial communities, which in turn can drastically affect marine animals. Characterizing the microbiome of each component of the complex network can help disentangle the dynamic interactions and individual influences of the host, microbes, abiotic and biotic factors.

Pacific oysters are the most widely farmed shellfish in the world\textsuperscript{1}; however, the industry is faced with hatchery production shortages due to mass-mortality events in post-metamorphic
oysters\textsuperscript{31–33}. The filter feeding behaviour, rapid development, and variable hatchery rearing conditions have made identifying the cause(s) of mortality challenging. In this study, using a high-through amplicon-sequencing approach, the microbiome of post-metamorphic oysters was characterized and monitored during the early hatchery rearing stage. In addition, the microbiome of oysters experiencing mortality was compared with ‘healthy’ oysters and oysters from a different farm site, to determine which microbial taxa occurred across all samples, and which were only associated with specific conditions or locations.

1.1 Biology of Pacific oyster (\textit{Crassostrea gigas})

Pacific oysters belong to phylum \textit{Mollusca}, class \textit{Bivalvia}, order \textit{Ostreoida}, family \textit{Ostreidae}, genus \textit{Crassostrea} (Thunberg, 1793)\textsuperscript{1}. East Asian native \textit{C. gigas} was introduced in 1903 to the west coast of the United States and in 1912 to British Columbia (BC), Canada, because of their resilience to wider ranges of environmental parameters and diseases than the native North American oysters, \textit{C. virginica} (the Atlantic or Eastern oyster) and \textit{Ostrea lurida} (the Olympia oyster)\textsuperscript{34,35}. \textit{Crassostrea gigas} has become the most prolific and abundant oyster species in the intertidal zone in the west coast of North America\textsuperscript{34}, as well as the dominant oyster species in aquaculture world-wide\textsuperscript{1}.

During the spawning season, Pacific oysters discharge gametes into the water and fertilization occurs within 10 to 15 hours\textsuperscript{34}. The fertilized eggs develop into planktonic larva (known as D-larvae) which drift in the upper layer of water column for 20 to 30 days; the larvae (0.30 mm) then settle (also known as “setting” or “spatting”) on hard or soft intertidal substrates or in shallow subtidal areas and metamorphose into their adult-like morphology\textsuperscript{36}. At this stage, the oysters are called spat, and range in size from 1 to 3 mm. The oysters spawn annually in July to August when the water is warmer; the relatively warm and brackish waters of the Strait of
Georgia (SoG) favour both spawning and rapid growth of Pacific oysters\textsuperscript{34,37}. In addition, in the summer upwelling in the SoG caused by northwesterly winds brings up cooler and nutrient-rich deep water\textsuperscript{38}, making the area ideal for oyster aquaculture\textsuperscript{34,37}. With optimal temperature and salinity, Pacific oysters can reach 10 to 15 cm in size after 2 to 4 years\textsuperscript{36}. Once mature, adult oysters can pump up to 7.3 L·h\textsuperscript{-1}·g\textsuperscript{-1} of their dry tissue weight, filtering particle sizes between 5 and 10 μm\textsuperscript{39}. Pacific oysters can live in a broad range of temperatures (8-22°C) and salinities (24-28 ppt) making them ideal for farming under a wide range of conditions\textsuperscript{1,34}.

Not only are Pacific oysters ideal shellfish for aquaculture, in the wild they can form dense reefs that can engineer surrounding water quality, providing habitats for various marine organisms and shape the local ecosystem\textsuperscript{36}. As a result, there is growing interest in using bivalve reefs to remediate estuarine eutrophication utilizing their potential role in biogeochemical cycles, such as nitrogen cycling\textsuperscript{40-42} and sulfur-oxidation\textsuperscript{43}. It has been reported that the sediments beneath a suspended oyster farm had higher denitrification rates, production of labile organic matter, and increased Fe\textsuperscript{2+} availability compared to control sediments\textsuperscript{42}. Similarly, sediments within mussel and oyster reefs in the Wadden Sea harboured bacteria that remove sulfide and subsequently generate acid\textsuperscript{43}. The exact mechanism and effect of denitrification processes or sulfur oxidation by oysters are yet to be fully understood. However, a study based on amplified 16S rRNA target gene sequences found taxa related to those known to have genes that encode proteins such as nitrate reductase and nitrous oxide reductase, suggest that oysters may be hotspots for denitrification\textsuperscript{41}.

\textbf{1.2 Pacific oyster aquaculture in British Columbia}

Pacific oyster aquaculture in Canada started on Vancouver Island (BC) in 1910, when oysters were imported from Japan\textsuperscript{37,44}. Continued imports from Asia resulted in widespread
natural-spawning of *C. gigas* populations in the SoG, and by the 1930s *C. gigas* was the most harvested intertidal shellfish in the province\(^{34}\). The development of larvae production became a major turning point in oyster aquaculture by allowing production of massive numbers of high-quality larvae as ‘seed stock’\(^{34}\). Larvae production further influenced the infrastructure of oyster aquaculture operation by overcoming shortages of adult spawning oysters in the wild.

Like other types of aquaculture, oyster aquaculture operates in two units; the hatcheries produce larvae or ‘seed stock’, and nurseries grow the seed stock until it reaches desirable sizes for wholesalers or consumers. As such, growth and survival of larvae in hatcheries is critical for providing spat for nurseries. To obtain oyster larvae, broodstock is first collected from naturally spawning reefs distributed along Pendrell, Pipestem and Baynes Sounds, and Lasqueti Island in BC. The gametes of the broodstock are strip-spawned (i.e. reproductive organs removed and artificially fertilized). This mass fertilization step is also known as batch culture, and usually produces up to 200 million larvae that are reared in a 40,000 L tank. Once the fertilized embryos reach the D-larva stage, the batch is transferred to a flow-through culture tank in which continuously flowing seawater that has been treated by sand and 0.2 µm filtration supplies feed from the bottom\(^{45}\). Debris is removed using a “saltplug” where saturated brine solution is fed into the bottom of the tank allowing live larvae to swim up while moribund stay at the bottom for drainage\(^{45}\). Temperature is maintained at 21°C and pH adjusted to ≥8 with CaOH, CaCl or NaOH. The flow rate is maintained so the entire volume of the tank is replaced every three days\(^{45}\).

When the shell length of the D-stage larvae reaches about 200 to 300 µm, they are transferred to a ‘setting’ or nursery tank where they attach to substrate and metamorphose into sessile juvenile oysters, also referred to as “spat” or “seed”. Upon metamorphosis, the spat grow
rapidly from less than 1 mm during the setting stage to 3 mm within a couple of weeks. The hatchery operators either continue growing the spat at the 3 mm size point in the flow-through system or sell them to nurseries where the spat are grown under more natural conditions. One common nursery method is the Floating Upweller System (FLUPSY), in which nutrient-rich deeper ocean water is “upwelled” to the floating rafts of growing spat.

Worldwide, aquaculture has the highest growth rate of any food production system, and shellfish aquaculture production reached 25M tonnes per year in 2012. Within the 25M tonnes, oyster production comprised 4.5M tonnes (valued at US $3.7 billion). In Canada, in 2016, more than half of the 102.325 tonnes of total farmed seafood produced was grown in BC (Statistics Canada 2017), more than that of the other six provinces combined, and had a final wholesale value of $32.5 million (BC Ministry of Environment). Production in BC is almost exclusively C. gigas, which in 2010 was 89% by volume, and 78% by value at harvest.

1.2.1 Mass mortality events in C. gigas spat contributing to seed stock shortages

Due to the nature of the mass production in aquaculture, mortality events have been reported throughout the oyster aquaculture history. The mortality events have mainly affected adult oysters and other oyster species however, in past decades, there has been growing reports of mass mortality in Pacific oyster spat and juveniles. Hatcheries in BC are one of the provinces that continue to experience mortality events of oyster spat. These mortality events have led to seed-stock shortages and problems for production in nurseries, leading to a 12% production drop in BC since 2003. In 2013, 30 out of the 34 respondents to a survey by the BC Shellfish Growers Association indicated not being able to acquire all of the seed stock requested, and acknowledged the problem of a lack of high quality and reliable sources of seed stock. In June 2018, the BC government started to provide short-term relief funds for oyster growers to
purchase seed stock to support continuity of production; however, this did not solve the problem of the lack of healthy seed stock.

The shortage of seed stock is especially problematic in Alaska (AK) where Pacific oyster aquaculture contributed 95% of the total value from state aquaculture sales in 2014\textsuperscript{47}; yet, as of 2014 oyster seed and larva could only be obtained from out-of-state hatcheries and nurseries, such as in Oregon and Washington State. Ongoing expansion of AK oyster aquaculture is faced with the backdrop of oyster-seed shortages; for example, in 2014, AK hatcheries only acquired 20\% of the oyster larvae and 38\% of the juveniles requested\textsuperscript{47}. The lack of Pacific oyster seed-stock limits the ability of industry expansion in areas such as AK that heavily rely on out-of-state seed stock and influences the growth of local communities and economic benefits to stakeholders.

Mortality events have occurred across all ages and production stages in farmed Pacific oysters, some of which were due to pathogens\textsuperscript{3,4,46,48}. One of the diseases of farmed Pacific oysters on the West Coast is the Protistan parasite \textit{Mikrocytos mackini} (Denman Island Disease), first reported in the 1960s in Vancouver Island\textsuperscript{49}. Another parasite associated with \textit{C. virginica} mortality events in 2007, \textit{Haplosporidium nelsoni} (Multinucleate Sphere Unknown X, MSX)\textsuperscript{50,51} was detected in a few \textit{C. gigas} from a grow-out facility in BC, Canada\textsuperscript{52}. Other bacterial pathogens such as \textit{Nocardia crassostrea} (Nocardiosis)\textsuperscript{33} and viral agents such as Oyster Herpes viruses (OsHV) have not been reported in BC or AK\textsuperscript{53}.

1.3 The Pacific oyster microbiome

As one of the most widely cultured oysters in the world, there has been a growing effort to characterize the Pacific oyster microbiome. This is in part due to global mortality events occurring across all life stages of Pacific oysters in both natural-spawning reefs\textsuperscript{2,33,54} and
farms\textsuperscript{3,4,32,55–57}. The studies can also lead to better understanding Pacific oysters as filter feeders and their effects on biogeochemical cycles\textsuperscript{42,43}. The majority of studies on the Pacific oyster microbiome have focused on patterns of microbiome dynamics related to temperature\textsuperscript{9}, spatio-temporal factors\textsuperscript{58}, tissues examined\textsuperscript{8}, host-genetics and antibiotic stressors\textsuperscript{8,54} (Table 1.1). Until culture-independent high-throughput sequencing such as 454 Pyrosequencing\textsuperscript{6} or Illumina MiSeq\textsuperscript{4,8} became available, previous microbiome studies used cloning, restriction fragment length polymorphism (RFLP)\textsuperscript{7,59}, and denaturing gradient gel electrophoresis (DGGE)\textsuperscript{60}, which have relatively lower species taxonomic resolution and scales of sequence output\textsuperscript{61,62} (Table 1.1). Nonetheless, common bacterial taxa were found in oysters, some of which were specific to Pacific oysters\textsuperscript{6}, specific tissues\textsuperscript{8}, and life stage\textsuperscript{5}.

Despite differences in sequencing approaches, sample processing regimes, and sample selection criteria, few common microbial taxa at the phyla level were associated with Pacific oysters and other oyster species (Table 1.1). Microbial composition and abundances of bacteria at the phyla level taxa among oysters were associated with examined tissue, life stages, temperature, sample location, and potentially filter-feeding behaviour, as some taxa occur across bivalve species and other filter feeders (Table 1.1). The most abundant phylum and class in other studies on \textit{C. gigas} were \textit{Proteobacteria} and \textit{Betaproteobacteria}\textsuperscript{5,59}, with the phyla \textit{Bacteroidetes}, \textit{Firmicutes}, and \textit{Actinobacteria} differing in relative abundance depending on life stage\textsuperscript{6,9,54}. \textit{Bacteroidetes} was higher in relative abundance in spat\textsuperscript{6}; whereas, \textit{Actinobacteria} was predominant in adults\textsuperscript{6}. \textit{Vibrio} spp. belonging to \textit{Gammaproteobacteria} was also commonly associated with other oyster species\textsuperscript{5}, as were other \textit{Proteobacteria} and \textit{Bacteroidetes}, with the latter two also being abundant in the surrounding marine environments\textsuperscript{7,63}. The commonly associated phyla across different oyster and bivalve species can be attributed to their residing
habitats and differing functional roles including breaking down filtered food, nitrogen fixation, and host-specific symbionts (e.g. *Bulkholderia cepacia* potentially protects against *Vibrio alginolyticus* and *V. harveyi*)\(^5,7\). Moreover, inferring functional roles of identified microbiomes among shellfish can assist in classifying the “core” versus “transient” populations of microbes\(^41,64\).

A shift in the microbiome has been observed for different life stages of *C. gigas*. For instance, pyrosequencing of 16S rRNA gene amplicons showed that the diversity of the microbiome was higher in spat than in adult oysters\(^6\), although the results varied among studies\(^5\). This difference in diversity may be due to changes in selective food ingestion with the maturity of the gastrointestinal tract of oysters, akin to a pattern observed in developing cod (*Gadus morhua*) larvae\(^65\). However, studies on oyster-associated microbiomes have targeted different tissues, locations and times, and used different processing, sequencing and analytical methods making it difficult to interpret observed differences in oyster microbiomes with developmental stage (Table 1.1).
<table>
<thead>
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<td>Alpha-diversity and Temperature Correlation</td>
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Table 1.1. Summary of previous microbiome studies done on Pacific oysters. *Proteobacteria* was reported in all the studies as the most abundant phyla regardless of the tissue types, location, identification methods, and 16S rRNA regions. The ‘Abundant phyla’ were defined by relative proportion compared to other phyla and environmental samples. For some studies, *Proteobacteria* and *Bacteroidetes* together made up >90% relative abundance of microbiome composition. DGGE=denaturing gradient gel electrophoresis; TGGE=temperature gradient gel electrophoresis; T-RFLP=terminal restriction fragment length polymorphism; g=genus; c=class; f= family.
1.3.1 The microbiome of farmed Pacific oyster spat that experienced mass mortality

Although numerous abiotic\textsuperscript{67,68} and biotic factors were linked to mass mortality in \textit{C. gigas}, none have been identified as a direct aetiological cause of mass die-offs across the world. As a result, the concept of a single pathogenic agent has been gradually replaced by one of complex interactions among many microbes; thus, constituting a pathobiome\textsuperscript{69} that can either directly or indirectly affecting oyster fitness. The first step in understanding the pathobiome is to characterize the microbial composition and relative abundance of each taxon, and compare it to the microbiome of a healthy group\textsuperscript{22,70}. Similarly, correlating taxa in the microbiome with mass mortality of oyster spat involves identifying the microbes associated with spat experiencing mortality\textsuperscript{71} and determining how it differs from the microbiota in healthy spat\textsuperscript{4,58}.

There is no single definition of the core microbiome but it is broadly defined as microbial taxa that are consistently associated with given ecological or biological habitats\textsuperscript{22,64}. In the context of host-microbiome, the core microbes can be considered as the meta-organism or “holobiont” of the host\textsuperscript{72–74}. The core microbiome can be categorized into two groups based on the extent of direct functional and fitness influence the host and microbes have on each other. For instance, symbionts can directly be involved in metabolic processing or out-compete pathogens\textsuperscript{75,76}. The other groups of core microbes that may not cause direct functional influence on hosts, can be more transient in nature (e.g. present only in one stage of life cycle or particular environmental conditions)\textsuperscript{77}, spatially variable (both geological and within the hosts), and occupy the host as a functional niche\textsuperscript{58,72}. For this thesis, the latter definition of core microbiome was used to describe oyster-associated microbiomes. Across different microbial communities, the core taxa can be identified by several modes of filtering and processing of operational taxonomic units (OTUs); this includes tallying of shared OTUs based on presence/absence data,
OTUs with similar frequency and relative abundances, closely related OTUs in which functional roles can be carefully inferred when there is an overlap in functional capabilities within the lineage, persistent OTUs that are detected across examined microbiomes from various times and sites\textsuperscript{78} and network analysis of co-occurring OTUs that can provide information on potential biological activities among microbes and predict microbiome characters of similar habitats\textsuperscript{22,64,79}. The current study characterized core microbiome by tallying persistent OTUs of similar frequency and relative abundance patterns in all oyster samples examined.

Characterization of the core microbiome opens doors for future studies that can tackle questions involving functional and metabolic relationships between the microbes and the hosts, as the microbes can closely interact with the hosts’ cellular and physiological functions\textsuperscript{80}. One notable example of a functional core microbe can be found in *Euprymna scolopes*, the Hawaiian bobtail squid\textsuperscript{81}. Part of the female reproductive system in this squid is called the accessory nidamental glad (ANG); it contains symbiotic roseobacteria taxonomically classified as *Leisingera* sp. JC1, which coat the eggs when laid. Genome sequencing and metabolite assays of the symbiont revealed that it may have antimicrobial activity and protect the eggs from pathogens and fouling agents. Hence, the core microbiome can be tightly linked to host evolutionary process\textsuperscript{82}. Along with hosts, the associated microbes are under natural selection, genetic drift and mutation leading to changes in the composition, abundance and diversity of microbes within the changing host ‘environment’ (the hologenome theory of evolution)\textsuperscript{73}. Hence the interaction between hosts and their microbiomes are interdependent and any change can have drastic effects on host fitness and the integrity of the microbiome. Dysbiosis is an example of a disturbed microbiome negatively influencing host fitness by becoming virulent (e.g. through
horizontal gene transfer) or losing mutualistic symbiotic functions (e.g. aid in metabolic processes or resource competition against pathogens)\textsuperscript{83,84}.

The main challenge in studying the host-associated microbiome is distinguishing resident microbes from transient, opportunistic or saprophytic microbes. The challenge is further complicated since the ‘core’ microbiome differs among tissues, life stages, species and growth environment of the host\textsuperscript{22,72}. In addition, transient microbes can become part of the core microbiome in later life stages of the host, or dysbiosis may allow transient microbes to become pathogenic\textsuperscript{83,85}. Such dynamics between the host and its core or transient microbiome have been hinted at through studies showing population shifts of \textit{Vibrio} species in \textit{C. gigas}. During mortality events of \textit{C. gigas}, increased loads of \textit{Vibrio} species were preceded by possible increases in susceptibility to infection by a mortality-associated virus, Osterid Herpes Virus (OsHV)\textsuperscript{86,87}. The lack of a cell-line for \textit{C. gigas} makes experimental studies of the microbiome difficult, although some pathogen research has been done on ‘axenic’ and depurated oysters\textsuperscript{87–89}. Depuration does not completely rid oysters of microbes, and some studies suggest that the remaining \textit{Vibrio} species in depurated oysters can affect the interpretation of results from downstream experiments\textsuperscript{87–89}.

The first step in linking the microbiome of \textit{C. gigas} spat to mortality is to see how the core microbiome changes with time, and to see if temporal shifts in the microbiome of healthy spat differs from that in spat experiencing mortality. This will indicate if mortality is associated with specific changes in the microbiome of developing oyster spat.

\textbf{1.4 Microbiome dynamics in the early developing \textit{C. gigas} spat}

Over a few weeks, oysters go through rapid physiological, anatomical and behavioural changes as they transform from embryos to planktonic larvae and metamorphose into spat\textsuperscript{34}. 
Along with these developmental changes, associated microbiome of the oysters also shifts, as has
been shown by comparing microbiomes across different life stages (i.e. larva or spat vs. adult
oysters)\textsuperscript{6,7}. Yet, little is known about changes in the composition of the microbiome in
developing spat, making it difficult to determine if changes in microbial composition associated
with oyster mortality might reflect an emerging pathogen, or whether the changes are simply age
related.

Changes in the microbiomes of aquatic larva are attributed to their constant contact with
the environment and feed, which shapes the microflora in the developing gastrointestinal
tract\textsuperscript{26,90}. Knowing the ecological interactions of microbes with developing spat could influence
hatchery management by minimizing disease outbreaks\textsuperscript{26,65,90,91}. Due to the history of disease
outbreaks\textsuperscript{46,92} in \textit{C. gigas} aquaculture caused by protists (e.g. \textit{Mikrocytos mackini}\textsuperscript{49},
\textit{Haplosporidium} \textit{spp.}\textsuperscript{50}, \textit{Bonamia ostreae}), bacteria (e.g. \textit{Nocardia crassostrea}\textsuperscript{92}, \textit{Vibrio
spp.}\textsuperscript{39,89,93}), and viruses (e.g. OsHV\textsuperscript{94}), microbiome studies investigating mortality events have
focused on finding rare or candidate pathogens\textsuperscript{3,4,33}. Furthermore, based on shifts in microbial
composition and diversity between healthy and diseased animals, characterizing the pathobiome
(i.e. microbiomes characteristic of diseased animals) has become a common approach\textsuperscript{22,69}.

Rapid changes experienced by growing spat may affect the microbiome composition;
thus, untangling the cause of mortality in developing oyster spat may not be as simple as finding
taxa that are similar to known pathogens or pathobiomes. Hence, analysis of the spat microbiome
should consider diversity, composition, and dynamics which can affect the 1) stability which
often parallels hosts health and is defined as equilibrium state with a central attractor that
microbiome transitions toward upon disturbances\textsuperscript{95}, and 2) resilience that can be inferred from
microbial diversity and defined as the amount of disruption microbiome can tolerate before shifting to a different equilibrium state\textsuperscript{14,26,95}.

1.5 Contest of Research and Hypotheses

Pacific oyster aquaculture is a main contributor meeting global seafood demand, but mass mortality events in hatcheries have resulted in seed-stock shortages; this affects continuity in production and destabilizes the industry. As mass mortality in oysters is often associated with microbial disease, I investigated the microbiome of Pacific-oyster spat from hatcheries that experienced mortality events in 2014. By comparing the bacterial microbiomes of spat which did or did-not experience mortality, I identified taxa that were associated with mortality, and I followed shift in microbiome dynamics during spat development. As well, I identified the “core” microbiome in Pacific oysters that persisted across time, mortality events, location, hatchery and juvenile production stage, and age. I also analysed the microbiome of juvenile oysters from an Alaskan farm and compared the results with those from BC spat. The hypotheses were that 1) some prokaryotic taxa are shared among BC and AK oysters and comprise the “core oyster microbiome” and 2) that some taxa are only associated with mortality in BC spat.
Chapter 2: Results

2.1 Introduction

Native to Japan, the Pacific oyster (*Crassostrea gigas*) is the most widely cultivated oyster worldwide, meeting the rising global demand for seafood. In Canada, British Columbia (BC) contributed more than 60% to annual oyster production (8,797 tonnes) in 2016 (2016 Canadian Aquaculture Production Statistics, DFO). Despite the resilience of *C. gigas* to a broad range of environmental conditions, mortality events at all life stages are the main hindrance to growth in aquaculture of *C. gigas*. Mortality events during the post-larval (spat) stage has had a particularly detrimental effect on the industry by limiting the availability of spat for nurseries to start production batches. Spat or seed-stock shortages have been an ongoing challenge for Pacific Coast shellfish communities, including in Alaska (AK) where production solely relies on out-of-state seed stock importation. Despite previous studies investigating the effects of ocean acidification, temperature, pathogens, and farm practices, the cause of oyster mortality events often remains unknown.

Next generation sequencing (NGS) data have shifted a perspective of disease studies from large-scale mortality being caused by varying environmental parameters (e.g. pH, salinity, temperature and spatio-temporal factors) or a single pathogen, to a concept that mortality arises from complex interactions between the environment and the host microbiome, through a process called dysbiosis or microbiome disturbance. Dysbiosis can result in compromised host immunity against opportunistic pathogens or functional disruption of core microbes, some of which may be symbiotic. Although there is no single accepted definition of a core microbiome, in this study it is defined as a tally of shared operational taxonomic units (OTUs) with similar relative abundances across samples from different times and locations.
The approach to characterizing the microbiome of *C. gigas* has varied across studies in terms of the choice of tissues, sample processing methods (e.g. depuration), geographic sources of broodstock, life stages, and sequencing methods. Furthermore, many studies have focused on microbiome dynamics in adult tissues collected from various grow-out sites with an emphasis on environmental factors such as temperature, pH, sites, and dynamics of candidate pathogens within the microbiome. Yet, mortality events in hatcheries have occurred while temperature, pH, and salinity were adjusted to optimal growth conditions, indicating that mortality was likely caused by other factors. However, the lack of studies on the microbiome during early oyster development (i.e. planktonic larvae to post-metamorphic spat) has made it difficult to infer the potential role of the associated microbiota makes in spat mortality.

Changes in gastro-intestinal microbiomes were observed in spat, juvenile and adult depurated Cortez (*C. corteziensis*), Kumamoto (*C. sikamea*) and Pacific (*C. gigas*) oysters, although resolution was limited by use of restriction fragment length polymorphism (RFLP) analysis and lower sequencing depth and coverage of pyrosequencing of amplified 16S rRNA due to generation of short reads. Studies on *C. gigas* and *C. sikamea* showed that in post-larval stages the relative abundance of *Bacteroidetes* and microbial diversity was higher. However, these studies did not examine changes in the microbiome of spat as they went through metamorphosis and physiological changes, or when they experienced a mortality event. The importance of characterizing the microbiome of the post-metamorphic spat is hinted at from studies on insects, amphibians and fish. For instance, the microbiome of newly metamorphosed southern leopard frogs (*Rana sphenoecephala*) harbored potential symbiotic bacteria that produced antimicrobial peptides against a chytrid fungus, while transient and
highly variable microbiomes were observed in *Lepidoptera* caterpillars\textsuperscript{103}. For oyster spat, research on microbial dynamics is limited.

Mortality events in farmed and wild oysters have been associated with infection by OsHV-1\textsuperscript{2,105} and some *Vibrio* spp.\textsuperscript{33,39,55}, as verified through viral particle isolation and infection assays\textsuperscript{96,106,107}. However, studies also indicate that mortality may result from polymicrobial diseases, in which commensal microbes such as *Vibrio* spp. acquire virulence\textsuperscript{39,108}, or an opportunistic pathogen invades stressed hosts that are experiencing dysbiosis\textsuperscript{84,87}. Similarly, initial infections by OsHV-1 could lead to an opportunistic bacterial infection that causes mortality\textsuperscript{87,109}. Yet, there have been no reports of OsHV-1 in BC; thus, local mortality events may be the result of emerging microbes or microbiome dysbiosis.

In the present study, culture-independent high-throughput sequencing (Illumina MiSeq) showed that the microbiomes of early-stage Pacific-oyster spat farmed in a BC hatchery changed between weekly samplings but did not show a developmental pattern in juvenile Pacific oysters farmed in AK, suggesting that the microbiome in farmed *C. gigas* may stabilize in the early spat stage. Using the Divisive Amplicon Denoising Algorithm (DADA2), which allows taxon to be resolved at a single nucleotide level\textsuperscript{110,111}, showed that microbial diversity and composition were not significantly different between cohorts of spat that experienced >70% or <50% mortality. However, the presence of mortality-associated taxa indicated potential dysbiosis. Shared operational taxonomic units (OTUs) between BC spat and AK juvenile oysters belonged to the family *Rhodobacteraceae* and phylum *Planctomycetes*, suggesting these taxa may be functionally important to Pacific oysters, despite different farm sites, production stages and sample times\textsuperscript{10,40,72}. The main findings of this study are that 1) early developing spat in the hatcheries showed developmental successions in microbiome composition, 2) the microbiomes
of BC spat that had been recently set in nursery tanks varied among few examined cohorts irrelevant of mortality events, 3) bacteria in the order *Alteromonadales* were commonly associated with BC spat that experienced mortality, and 4) frequently identified OTUs (as part of the core microbiome), some of which were identified as taxa that may oxidize sulfur and fix nitrogen, occurred among older BC spat and AK juvenile oysters.

### 2.2 Materials and methods

#### 2.2.1 British Columbia (BC) Pacific oyster spat farm sites and cultivation

The Pacific oyster (*C. gigas*) spat used in the project were provided by Island Scallops Ltd. (ISL) located in Qualicum Beach, BC, Canada (49° 19’ 29.64’’N, 124° 19’ 48.96’’W). The ISL hatchery broodstock was a collection of naturally spawning Pacific oysters from nearby areas such as Pendrell Sound (Desolation Sound on East Redonda Island), Pipestem Inlet (in Barkley Sound on Toquart Bay), and Lasqueti Island.

The hatchery production followed general FAO (Food and Agriculture Organization of the United Nations) guidelines starting with strip-spawning of the adult oysters. The larvae (up to 100 million) were grown in flow-through 40,000 L tanks with a three-day turnover time for the water. Once the larva metamorphosed, the cohort of spat was caught on >310 μm screens and transferred to a nursery tank; nine cohorts were used in the study. The tank water was adjusted to optimal conditions for spat growth (21°C, pH 8.1-8.2, salinity 25 ‰, 3.3 mg/L alkalinity) and filtered through sand and 5-μm filters. Spat between 400 and 1000 μm were collected from the nursery tanks, and the pH, temperature, salinity, alkalinity and nitrite levels of the incoming seawater and adjusted tank water were recorded.
2.2.2 Alaska (AK) juvenile Pacific oyster farm sites and cultivation

Juvenile oysters (2.09± SD 0.86 cm) were from a nursery in Halibut Cove, Alaska (59° 35’ 52”’N, 151° 13’ 30”’W) operated by Kachemak Shellfish Growers (KSG), and were grown from spat purchased in March 2016, from Whiskey Creek Shellfish Hatchery, Oregon; the broodstock were sourced from nearby Netarts Bay. The spat were grown in the KSG nursery until May 2016, and then transferred into a floating upwelling system (FLUPSY), which propels nutrient-rich water through the oyster rafts.

2.2.3 Sample collection, processing and DNA extraction

2.2.3.1 BC spat, water filter collections, and DNA purification

Nine cohorts of BC spat and seven water samples were collected during May to October 2014. The spat and water samples were collected weekly and the survival % recorded daily. To compare the microbial signatures between the spat and surrounding water, three replicates of 2L tank water were filtered onto 0.22-µm pore-size Durapore (Millipore Sigma, Burlington, MA) low-protein-binding PVDF membranes. Each filter was placed into a sterile plastic bag and stored at -80°C until processing.

Out of the nine BC-spats cohorts, four experienced >70% mortality at each time point and are referred to as ‘mortality (M)’ cohorts. The other five cohorts had normal 10-50 % mortality per time point and are referred to as ‘control (C)’ cohorts. The microbiomes of control and mortality cohorts that were set within two weeks of each other (except Pair 1), were compared in four pairs: Pair 1 containing BC cohort 1 and 5 (set date late May and late July 2014, respectively); Pair 2 containing BC cohort 13 and 14 (set date early July, 2014); Pair 3 containing BC cohort 15 and 16 (set date mid August, 2014); Pair 4 containing BC cohort 18, 19 with the set date early September, 2014 (and cohort 20 with the set date late September, 2014).
All the mortality events occurred within the first two weeks of the spat setting in the nursery tanks. For each mortality cohort, samples for microbial community analysis were collected at a minimum of three time points spanning the mortality peak (i.e. lowest survival % per time point; Fig 2.1), and are referred to as ‘before’, ‘during’ and ‘after’ the mortality peak. Each week, 100 µL or more of spat (representing 15 to 30 individuals) was collected in a 2-mL screw-cap cryovial and stored at -80°C until processing. The samples from the control cohorts were selected based on ‘days-since-set’ (represents age), so that control and mortality cohorts were comparable. BC Cohort 5 and 13 only two time points (no ‘during’ mortality peak samples) associated with mortality peaks were available for analysis.

Figure 2.1. Cumulative % survival of nine BC (ISL) spat cohorts grouped into four pairs, each containing the mortality and the control cohort. The mortality cohort is indicated by the blue line and the control cohort is indicated by the orange line. Each point (circle) on the lines represents the daily record of the survival percentage. Different colour-points on each line (triangle, diamond and square shapes) represent the date of that spat samples were collected for analysis. The grey line (BC Cohort 20) was included as another control cohort because it was set in the same month as BC Cohort 18 and 19. The number of larvae at Day 0 ranged from 10 to 30 million.

DNA for sequencing was extracted as outlined below. Using a sterile spatula or pipette, approximately 20 spat were transferred into a sterile 1.5-mL microcentrifuge tube containing two 3.2 mm diameter chrome steel beads (Bio Spec Products Inc., Bartlesville, OK). The spat were
homogenized in a Mixer Mill MM300 (Retsch GMBH, Haan, Germany) for 1 min with frequency at 1/30 s. At least 2 mg of tissue homogenate from each tube was transferred to a new microcentrifuge tube, and DNA extracted using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and per manufacture’s protocol, with an added lysozyme (10 mg/mL) lysis step after Proteinase K addition. DNA was extracted from the 0.22-µm pore-size membrane filters with a DNeasy PowerWater kit (Qiagen, Hilden, Germany), following the manufacturer’s protocol. DNA in the extracts was quantified with the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA).

### 2.2.3.2 AK juvenile oyster, water filter collections and DNA purification

Alaskan juvenile oyster (size 2-4 cm) samples and seawater from the FLUPSY (floating upweller system) were collected weekly from mid-September to November 2016 by staffs at Kachemak Shellfish Growers (KSG). Approximately 20-40 oysters were sampled into a Ziploc bag for each of the eight time points. The seawater from seven weekly time points was filtered onto 0.22-µm pore-size Durapore PVDF membrane Millex GV (Millipore Sigma, Burlington, MA) syringe filters mounted on sterile 30-mL BD syringes. The filter units were placed into Whirl-Pak bags (Millipore Sigma, Darmstadt, Germany) and stored at -20°C until shipped to the University of British Columbia (UBC), where they were stored at -80°C until processing. All samples were collected in duplicate.

For each time-point, gloves and a sterile spatula were used to shuck each of 15 oysters that were transferred onto sterile petri dishes, prior to DNA extraction as follows: Each oyster was homogenized in a mixer mill for 5 to 7 min at 1/30 s, with chrome steel beads. DNA extraction and quantification were the same as for the BC oysters and the water samples. For each time-point, 100 ng of DNA from 15 juvenile oysters was pooled and diluted in 100 µL of
PCR-grade water. To minimize rapid thawing of the frozen tissues, both the BC and AK samples were slowly thawed on ice and carried on dry ice between processing steps.

2.2.4 Library preparation

The sequencing libraries were prepared following the Illumina MiSeq 16S Metagenomic Preparation Guide with Nextera XT v2 Kit (Illumina, San Diego, CA) with minor modifications as detailed below. The V4-V5 (515F-926R) hypervariable regions of the small subunit (SSU) 16S ribosomal RNA coding gene were targeted for amplification\textsuperscript{112–116}. Conditions for PCR were denaturation for 5 min at 95°C followed by 34 cycles at 95 °C for 45 s, 50 °C for 45 s, 68 °C for 90 s and a final extension at 68 °C for 10 min\textsuperscript{113}. In a 25 µL reaction volume, the PCR mixtures contained 1 to 10 ng of DNA, 1X Q5 Reaction Buffer (NEB, Ipswich, MA), 0.4 µM barcoded forward and 0.4 µM reverse primers, 0.1U Q5 High-Fidelity DNA Pol (NEB, Ipswich, MA), 0.8 mM dNTPs, 1X BSA (100x), 2.5 mM MgCl\textsubscript{2} (Zhong et al., unpublished). The Index-PCR reaction was performed in a 25 µL volume containing 10 ng DNA, 1X Q5 Reaction Buffer (NEB, Ipswich, MA), 1.25 µL each of Nextera XT v2 Index 1 and 2 Primer, 0.1U Q5 High-Fidelity DNA Pol (NEB, Ipswich, MA), 0.4 mM dNTPs, 2 mM MgCl\textsubscript{2}. Amplicons were purified, and size-selected using Agencourt AMPure XP Beads (Beckman Coulter, Brea, CA). The libraries were quantified with the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and the Index attachment was verified using quantitative PCR (qPCR) with the SsoFast EvaGreen Supermix (BioRad, Hercules, CA) and KAPA DNA Standard (KAPA Biosystems, Boston, MA) on a C10000 Touch PCR block with a CFX 96W Reaction Module (BioRad, Hercules, CA). The libraries were pooled, and gel-purified in a 1.5 % agarose gel by excising the 560 base-pair (bp) band using a Zymoclean Gel DNA Recovery Kit (Zymo, Irvine, CA), following the manufacturer’s instructions. The pooled libraries were validated with a
Bioanalyzer High Sensitivity DNAchip (Agilent, Santa Clara, CA) and sequenced using 2 x 300 bp paired-end sequencing on the MiSeq platform (Illumina, San Diego, CA) at the BRC-Seq facility (UBC, Vancouver, BC).

### 2.2.5 Processing 16S rRNA amplicon sequence data

Raw-read data was demultiplexed and cleaned using Quantitative Insights into Microbial Ecology (QIIME 2 version 2018.6.0)\(^{117}\). Primers, low quality sequences (median quality (Phred) score < 29) and chimeras were filtered, and forward and reverse reads were merged using the Divisive Amplicon Denoising Algorithm 2 (DADA2)\(^{111}\), an algorithm QIIME2 is built upon. The output of the analysis was a tabulated list of features, each representing a biological sequence variant, generated by the \textit{de novo} error correction method of DADA2\(^{111}\). To assign taxonomy to each feature, feature-table data was trained with the SILVA reference database (release 132) with a 99 \% similarity threshold. Based on the feature-table, a sequence similarity tree was generated using \textit{de novo} multiple sequence alignment (MAFFT)\(^{118, 119}\). Features with long branches were searched using the Basic Local Alignment Search Tool (BLAST) and removed based on query length, e-value and hit identity\(^{120, 121}\). Mitochondria, chloroplasts, metazoan and single-frequency sequences were filtered from the feature-table.

Prior to the analysis, the frequency of features was rarefied to equal numbers across samples, capturing maximum sequencing depth without losing sample information. Rarefied features were then formatted into an OTU-table for further graphic visualization using R with the Phyloseq R package\(^{121, 122}\). The terms “OTU” and “features” are used interchangeably. To measure species diversity (Shannon-Wiener index) and evenness (Pielou’s evenness values) of the microbial communities, alpha diversity was computed using QIIME 2. To compare community composition between predetermined variables (e.g. locations, age and sample type),
the Bray-Curtis dissimilarity index was calculated and ordinated using Principal Coordinate Analysis (PCoA), and nonmetric multidimensional scaling (nMDS). To find taxa unique to each microbiome, the Local Contribution of Beta Diversity (LCBD) was calculated using the MicrobiomeSeq R package\textsuperscript{123,124} and Hellinger distance was calculated to quantify dissimilarity between microbiome structures based on OTU abundance. A high LCBD value in a sample within the defined group represents a marked difference from the average beta-diversity (i.e. uniqueness)\textsuperscript{123}.

To assess if specific OTUs are associated with mortality events, farm sites, and ages, indicator species analysis was performed with 999 permutations using Indicspecies R package\textsuperscript{71,125,126} to produce an Indicator Value Index (IndVal) of the associated species and a group representing different variables (i.e. farm sites, mortality cohorts)\textsuperscript{71}. The IndVal is calculated based on the species abundance and frequency in a designated group (i.e. mortality cohort) relative to the abundance and frequency in all groups. The statistical significance was computed using a permutation test (significance level, $p = 0.05$).

To find common taxa that were shared across all oyster samples, and to infer symbiotic and ubiquitous microbes, core microbiome analysis was conducted using the Microbiome package in R\textsuperscript{27,127}. The core taxa were generated by calculating the relative proportion of the OTUs, which are defined as those that exceed the set parametric prevalence threshold calculated in the Microbiome package (i.e. the level of prevalence across samples) at a given detection level (i.e. OTU abundance regardless of its prevalence in all samples). The core microbiota for all BC spat samples were calculated based on the absolute counts of the OTUs with a detection limit determined by setting the maximum abundances into logarithmic scales using $10^{\text{seq}(\log_{10}(1), \log_{10}(\max(\text{abundances(FeatureTable)}/10)))}$ function in the Microbiome package\textsuperscript{27,127}. 
2.3 Results

2.3.1 Sequencing reads summary and rarefication

To assess prokaryote microbiome communities of the oysters, the V4-V5 hypervariable region of SSU 16S rRNA gene was targeted. Prokaryotic DNA from farmed BC and AK oysters was amplified and sequenced using Illumina MiSeq 2 x 300 paired-end approach. The final pooled concertation of all sample amplicons (total 70 libraries) was 29.3 nM, 100 ng of each library. In total, 12 pM of the pooled library sample was loaded onto the flow cell along with a 10% PhiX spike, resulting in 39,473,320 raw reads of which 81% (34,473,320 reads) passed internal quality filtering and were used for downstream analysis. The demultiplexed forward and reverse reads were merged after removing primer sequences and trimming low quality (Phred score) ends, using DADA2. In total, 44,664 OTUs identified with a unique representative sequence, and using a de novo multiple sequence alignment, gapped columns or non-conserved regions of aligned sequences were filtered out. After filtering (i.e. removing eukaryotic signals, single-frequency features, PCR errors and chimeras), the total number of OTUs decreased to 40,261. To calculate the alpha and beta diversity metrics and generate ordination without sampling bias, the sequences were rarefied to 16,881 reads per sample, retaining 47.18% of the OTUs from all 70 samples, indicating deeper sequencing depth was needed to recover all of the microbial diversity.
2.3.2 Summary of the microbiome structure of BC oyster spat

2.3.2.1 Spat ages, but not mortality events were associated with significant changes in alpha diversity.

Out of 70 sample libraries, 47 were from the BC spat and filtered tank water, and yielded 55% (1,373,696) of the total sequence reads with a mean of 27,450 reads per sample. Out of 40,261 total OTUs, 54.5% were present in the BC samples libraries, and after read rarefication 61.5% of these OTUs were retained. In order of occurrence, the top five most common phyla across the BC spat and tank-water samples were *Proteobacteria, Bacteroidetes, Planctomycetes, Verrucomicrobia*, and *Patescibacteria*, while in the BC spat samples alone they were *Proteobacteria, Bacteroidetes, Planctomycetes, Patescibacteria* and *Chloroflexi* (Fig 2.2).

There were no significant differences in alpha diversity between mortality and the control cohorts within each pair (Table 2.1). The same result was observed, as well as for richness and evenness, without the pair groupings (Fig 2.3). When alpha diversity was compared by mortality timing (‘before’, ‘during’ and ‘after’) against the control cohort samples, significantly lower richness (measured by normalized OTU reads) was observed ‘before’ the mortality event compared to ‘after’ (Fig 2.4a). The richness difference between the ‘before’ and ‘after’ samples (Fig 4a) is likely an age effect since the three mortality-timing overlap with the spat age progression (in days).

To verify the age effect on alpha diversity, the spat samples were separated into eight age groups (A-H) and measuring from the first day the spat were set (Day 1) to 47 days old. Both
pair-wise ANOVA and Kruskal-Wallis tests showed significant differences in alpha diversity with age (Table 2.2).

Control (C) Cohort Mortality (M) Cohort  H  p-value  q-value
Pair 1  BC01 (n=4)  BC05 (n=3)  2.00  0.157  0.436
Pair 2  BC14 (n=5)  BC13 (n=4)  3.84  0.050  0.225
Pair 3  BC15 (n=3)  BC16 (n=3)  0.428  0.513  0.659
Pair 4  BC18 (n=7)  BC19 (n=6)  0.327  0.568  0.681

Table 2.1. Pair-wise Kruskal-Wallis test results comparing Shannon Index of the mortality and the control cohorts in the four pairs of BC spat. The BC20 (control cohort, n=4) was omitted in the table but showed no significant difference with BC18 and BC19 (p-value=0.131 and 0.201, respectively). ‘n’ indicates number samples used per cohort. The spat setting date for Pair 1 was late May and July for cohort 1 and 5 respectively; Pair 2 set date was early July 2014; Pair 3 set date was late July 2014; Pair 4 set date was early September 2014.
Table 3.2. Pair-wise Kruskal-Wallis test on the Shannon Index of the BC spat age groups. Only the test pairs with p-values < 0.05 are shown.

<table>
<thead>
<tr>
<th>AgeGroup</th>
<th>AgeGroup</th>
<th>H</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (n=7)</td>
<td>G (n=2)</td>
<td>4.20</td>
<td>0.040</td>
<td>0.334</td>
</tr>
<tr>
<td>C (n=8)</td>
<td>G (n=2)</td>
<td>4.36</td>
<td>0.037</td>
<td>0.334</td>
</tr>
<tr>
<td>D (n=6)</td>
<td>G (n=2)</td>
<td>4.00</td>
<td>0.046</td>
<td>0.334</td>
</tr>
</tbody>
</table>

Figure 2.3. Richness, alpha diversity, and evenness box plots with pair-wise ANOVA (Analysis of variance) for the four pairs of the mortality and control spat cohorts. Box plots of the nine BC cohorts organized by the mortality (Mort) and the control (Ctrl) cohorts. No significant differences (p-value >0.05) were observed between the diversity of mortality and control cohorts, regardless of the set time. The significance level was tested again with Kruskal Wallis pair-wise test (p-value = 0.058).

Figure 2.4. Alpha diversity box plot of BC spat grouped by (a) mortality timing and (b) age-group, with significance levels from pair-wise ANOVA indicated. (a) Alpha diversity based on the timing of mortality, showing significant differences in richness between after and before the mortality peak (p-value<0.01). ‘No’ indicate samples from the control cohorts. (b) Alpha diversity measure of the BC spat organized by age-group. The letters for each AgeGroup represents the number of days since the set date, and are as follows: A=0-5 days old; B=6-11 days old; C=12-17 days old; D=18-23 days old; E=24-29 days old; F=30-35 days old; G=36-41 days old; H=42-47 days. (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001)
2.3.2.2 The microbiome of the early developing spat separate temporally

To assess the similarity in microbial composition among oyster developing spat, the Bray-Curtis dissimilarity was calculated and visualized using Principal Coordinates Analysis (PCoA) (Fig 2.5a). To visualize the effects of mortality timing and spat age (i.e. days since set in the nursery tanks) on beta diversity, non-parametric multidimensional scaling (nMDS) was used (Fig 2.5b). The analyses showed that changes in beta diversity were primarily related to age and not to mortality (Fig 2.5b). There was no clustering in the PCoA plot when the ordination was visualized by cohorts, sample dates or mortality occurrence. Moreover, when environmental parameters (e.g. pH, temperature, salinity, alkalinity, nitrite) were fitted onto the nMDS ordination, only age was correlated with beta-diversity (Appendix A).

Changes in microbial composition with spat age were evident at both the phylum and family levels (Fig 2.6). Sequences assigned to the phylum Bacteroidetes prevailed in the 0 to 5 d age class (A) and were superseded by Proteobacteria as the spat developed, except for the 36 to 41 d (G) age class, which was again dominated by Bacteroidetes (Fig 2.6). At the family level, the Bacteroidetes was dominated by OTUs assigned to the Flavobacteriaceae in all age classes. Within the Proteobacteria, OTUs associated with the family Rhodobacteraceae initially dominated, but shifted to those in the Nitrosomonadaceae as the spat developed (Fig 2.6). Operational taxonomic units assigned to the phylum Patescibacteria were much less abundant, and at their peak (0 to 5 d) only represented 7% of the total reads; whereas, sequences belonging to the phylum Planctomycetes were relatively more abundant (up to ~20%) in spat from 6 to 29 days old (groups B-E). Sequences assigned to the phylum Chloroflexi were observed in 18 to 41 day spat (groups D-G) but were always a minor component (<2%) of the total reads. In summary, the phylum Chloroflexi gradually increased in relative abundance, Patescibacteria...
decreased, and *Planctomycetes* peaked between 6 and 23 days, as the spat aged. A progressive shift in OTUs assigned at phyla and family levels was observed across weekly time points, while some OTUs were more abundant in specific age-groups.
Figure 2.5. The beta diversity quantified by Bray-Curtis dissimilarity. (a) Beta diversity by age group visualized by PCoA ordination shows a transition of beta diversity along the two axes. Darker colours from A through H represent older oysters. (b) Changes in the microbiome relative to mortality events and age of the spat, visualized by nMDS. The figure shows that the microbiome differed more strongly by age-group (colours and letters) than by whether-or-not mortality had occurred (symbols) (Stress=0.128). Samples collected at the same time relative to mortality events are connected by lines and coloured by age-group. The letters indicate the age group as follows: A=0-5 days, B=6-11 days, C=12-17 days, D=18-23 days, E=24-29 days, F=30-35 days, G=36-41 days and H=42-47 days since the nursery set date.

Figure 2.6. Shifts in relative abundance of sequences assigned to families for the top five phyla (age-groups A-H). The height of each bar represents the number of sequences assigned to a phylum for each age-group. The number of sequences assigned to a family are represented by different colours. The unlabeled top colour indicates unresolved family-level taxonomy, while black indicates other families that are not listed. Sequences assigned to the Bacteroidetes and Patescibacteria dominate the younger age classes (A and B); whereas, except for age-class G, Proteobacteria sequences dominate the older age groups. Planctomycetes sequences are most prominent in the middle age-groups (B to D), while Chloroflexi sequences are more abundant in later age-groups (E-G), but are always a small proportion of the total. Letters indicate time from set, as follows: A=0-5 days, B=6-11 days, C=12-17 days, D=18-23 days, E=24-29 days, F=30-35 days, G=36-41 days and H=42-47.
To identify taxa that were contributing to the significant (p < 0.05) difference in beta diversity by age group, a Local Contribution to Beta Diversity (LCBD) test was conducted with the Hellinger dissimilarity coefficient method\textsuperscript{123,124}. OTUs assigned to the genus *Nitrosomonas* gradually increased, while those from the family *Flavobacteriaceae* decreased, as the oysters aged (Fig 2.7a); whereas, those from the families *Rhodobacteraceae* and *Rubinisphaeraceae* (genus *Fuerstia*) were most abundant in spat that were 1 to 3 weeks old. In addition, each age group seems to harbour different sequence variants (i.e. different OTUs) of *Flavobacteriaceae*. For instance, the OTU assigned to the genus *Dokdonia* 171 was more abundant in later age groups; whereas, another OTU assigned to the variant *Flavobacteriaceae* 1387 was more abundant in younger spat (Fig 2.7a). Different OTUs at the family level were associated with each age-group of spat; *Flavobacteriaceae* in the youngest group followed by *Nitromonadaceae* and *Rhodobacteraceae* in the older age groups.

The LCBD test was repeated on spat organized into pairs in which mortality occurred or did not occur (Pairs 1-4; Fig 2.7b). The shift in taxa abundance with spat age also occurred within the mortality and control cohorts, while all mortality cohorts had a higher proportion of OTUs assigned to *Flavobacteriaceae* 1387 (dark brown), relative to its control pair.
Figure 2.5. Local Contribution to Beta Diversity (LCBD) analysis of BC spat organized by age-groups and mortality pairs, showing the top 21 taxa that contributed to changes in beta diversity. (a) BC spat samples from all 9 cohorts were organized by age groups (A–H), irrespective of mortality events. The y-axis ‘Value’ indicates the proportion of each taxa within the sample. Letters indicate time from set, as follows: A=0–5 days, B=6–11 days, C=12–17 days, D=18–23 days, E=24–29 days, F=30–35 days, G=36–41 days and H=42–47. (b) The BC spat cohorts grouped into four pairs (Pair 1–4), each containing the mortality (M) and the control (C) cohort. Pair4.5C indicates the BC20 cohort that was set >2 weeks from the Pair4 (M & C) set date. The gradient-filled wedges indicate older spat (in days) with greater thickness of the wedge. Shifts in taxonomic composition with age were also observed within mortality pairs. The mortality (M) cohorts in all four pairs had a higher proportion of the Flavobacteriaceae 1387 variant compared to its control (C) pair. The diameter of the circles above each sample name indicates relative uniqueness (LCBD value) compared to average beta diversity within each group. Most samples from the younger age group had higher LCBD values as shown by larger diameter of the circles in younger age groups in (a) and (b) within the Pair1, Pair2M, Pair3, Pair4C.
2.3.2.3 Core taxa across the BC spat differ by prevalence and abundance

The core microbiome was defined following the criteria that an OTU had to be prevalent (i.e. in >70% of total samples) and abundant\textsuperscript{27} (>700 detection threshold calculated from sequence reads from total samples). I found that the core microbiome was comprised of taxa in the following genera: \textit{Fuerstia} (phylum \textit{Planctomycetes}), \textit{Gaetbulibacter} (\textit{Bacteroidetes}), and \textit{Roseobacter} (\textit{Proteobacteria}) (Fig 2.8). Abundant genera with >700 detection threshold were \textit{Psychroserpens}, \textit{Dokdonia}, \textit{Leucothrix} (\textit{Proteobacteria}), \textit{Bernardetia} (\textit{Bacteroidetes}), \textit{Acanthopleuribacter} (\textit{Acidobacteria}), \textit{Loktanella} (\textit{Proteobacteria}), \textit{Pesicirhabdus} (\textit{Verrucomicrobia}), \textit{Rickettsia} (\textit{Proteobacteria}), \textit{Roseibacillus} (\textit{Verrucomicrobia}), \textit{Tennacibaculum} (\textit{Bacteroidetes}), and \textit{Winogradskyella} (\textit{Bacteroidetes}). Prevalent taxa (some of which occurred > 90% of total samples) were \textit{Sedimentitalia}, \textit{Roseovarius}, \textit{Roseobacter}, \textit{Nautella}, \textit{Lentillitoribacter}, \textit{Jannaschia} (\textit{Proteobacteria}), \textit{Gaetbulibacter}, and \textit{Fuerstia}.

Although the core microbiome was defined as OTUs that were present in relatively high abundance in more than 70% of the total samples examined, it may not capture members of the core microbiome that are transient\textsuperscript{128}, or (symbiotic and mutualistic) microbes that occur in low abundance\textsuperscript{129}. 
Figure 2.6. The core taxa of the BC spat visualized by OTU prevalence and abundance. The arrows indicate taxa that were prevalent across the samples with a detection threshold >700 (abundance, denoted by * on the x-axis with a dashed-line). These are the genera *Winogradskyella, Sedimentitalea, Roseovarius, Roseobacter, Rickettsia, Psychroserpens, Poribacter, Nautella, Litoreibacter, Lentilitoribacter, Jannaschia, Gaetbulibacter, Fuerstia, Bdellovibrio, and Aquibacter, Psychroserpens, Nautella and Fuerstia*, which were both prevalent and abundant in the BC spat samples. Some core microbiome genera (e.g. *Dokdonia*, red arrow and *Leucothrix*, black arrow) were not present in all BC samples, but were in high abundances from total OTU reads combined from all the samples.
### 2.3.2.4 The order Alteromonadales and the genus Roseovarius were associated with mortality

To identify microbial taxa only associated with mortality timings (‘before’, ‘during’, and ‘after’ mortality peaks), Multilevel Pattern Indicator Species analysis was done using the Indicspecies package in R (significance level, alpha = 0.05)\(^1\)\(^2\)\(^3\)\(^4\)\(^5\). The OTUs associated with samples from the ‘before-mortality’ (BM) peak were the genera *Marinagarivorans*, *Oleiphilus*, *Psychrobium* and “uncultured bacteria” (Table 2.3). The families *Alteromonadaceae*, *Rhodobacteraceae*, and the class *Ignavibacteria* were associated with ‘before’, ‘during’, and ‘after’ mortality peaks. No indicator species were only associated with after the mortality peak; however, an OTU assigned to an uncultured bacterium belonging to the *Planctomycetes* occurred in samples collected ‘during’ and after ‘mortality’ peaks. To determine if there were any OTUs that were only associated with the healthy cohorts, the indicator species analysis was repeated however, no OTUs were identified.

<table>
<thead>
<tr>
<th>Mortality timing</th>
<th>List of indicator species (Significance codes for p-values: 0.0001 ***; 0.001 **; 0.01 *)</th>
</tr>
</thead>
</table>
| BM (5)           | • **Gammaproteobacteria**:Cellvibrionales;Cellvibrionaceae;Marinagarivorans***<br>• **Gammaproteobacteria**:Oceanospirillales;Oleiphilaceae;Oleiphilus*<br>• *Bacteroidia*:Sphingobacterales;NS11 12 marine group;uncultured bacterium*<br>• *Gracilibacteria*:JGI 0000069 P22; bioreactor metagenome*<br>• **Gammaproteobacteria**:Alteromonadales;Shewanellaceae; *Psychrobium*<br>BM+DM+AM (3) |<br>• Gammaproteobacteria;Alteromonadales;Alteromonadaceae *<br>• *Ignivibacteria*:OPB56;uncultured.bacterium*<br>• **Alphaproteobacteria**:Rhodobacterales;Rhodobacteraceae;Roseovarius*<br>BM+DM+AM (3) |<br>• Gammaproteobacteria;Alteromonadales;Alteromonadaceae *<br>• *Ignivibacteria*:OPB56;uncultured.bacterium*<br>• **Alphaproteobacteria**:Rhodobacterales;Rhodobacteraceae;Roseovarius*<br>Table 4.3. Indicator species analysis on different mortality timing (‘before’, ‘during’, and ‘after’ mortality peak). BM=before mortality peak; DM=during mortality peak; AM=after mortality peak. Except for the phylum (*Planctomycetes*) in AM+DM, taxa are listed by class;order;family;(genus if applicable). The brackets following each group name indicate the number of identified OTUs.
2.3.3 The microbiome of AK juvenile oysters did not significantly change with age

To determine if the AK oysters have temporal shifts in microbial composition and abundances, the sequence data for the Alaskan juvenile oysters were subsampled and analyzed separately from the BC spat samples. Excluding extraction blanks and PCR controls, 18 of the 70 libraries were of AK juvenile oysters (n=11) and FLUPSY water samples (n=7). Approximately 29% of the total sequencing reads and 45% the total features represented AK samples. For diversity calculations, the sequence reads were normalized to 20,026, retaining 50% of reads for the 18 samples. The top five phyla across the AK oyster samples were Proteobacteria, Planctomycetes, Bacteroidetes, Verrucomicrobia and Actinobacteria.

There was no significant change in diversity indices (richness, Shannon Index and Pielou Evenness) when the samples were grouped by cohorts (organized by the month when the oysters were sampled) and compared with the FLUPSY water samples (Kruskal-Wallis test for Shannon Index; p-value= 0.587; 0.258, respectively). To investigate potential changes in the microbiome with age, the AK juvenile oysters were grouped into four age-groups based on the days from the nursery tank stage. Unlike the BC spat samples, no age-related shifts in diversity indices or beta diversity were observed in the microbiomes of the AK oysters (Kruskal Wallis p-values for richness, Shannon Index and Pielou = 0.665, 0.336 and 0.149, respectively; ANOSIM statistic R=0.256; significance=0.06). However, there was a gradual increase in the relative abundance of Planctomycetes and a decrease in Verrucomicrobia, although the increase did not contribute to differences in overall beta-diversity (Fig2.7b). The LCBD test was conducted on the AK oysters by the four age-groups to identify key taxonomic shifts in microbial composition; two variants of Rubritaleaceae were present in higher proportions in earlier sample sets, and the proportion of
Pirellulaceae (phylum Planctomycetes) was higher in the older oysters (Fig 2.9a). Similarly, the contribution of families to the top phyla changed among age groups (Fig 2.9b).

Figure 2.7. Local Contribution to Beta Diversity (LCBD) analysis, and abundance of top five phyla in the AK juvenile oyster microbiome, organized by four age-groups. (a) LCBD test showing 21 most abundant taxa that contributed to differences in beta diversity when organized by the four age-groups. Different genera in the family Pirellulaceae increased with the age of the oysters, while Rubritaleaceae (Verrucomicrobia) decreased. (b) Relative abundance of families in the top phyla by age-groups, showing increasing relative abundance of Pirellulaceae and decrease in Rubritaleaceae, except the age-group AD. The abundance of reads was generated using the top 40 OTU normalized reads. AA = 135-155 days; AB = 156-176 days; AC = 177-197 days; and AD = 198-218 days since setting.
2.3.4 Taxonomic composition of the AK and BC oyster microbiome was markedly different

To determine differences in the microbiomes of spat from BC and juvenile oysters from AK, microbial diversity and composition were compared between the two groups. The alpha diversity in the AK oyster samples was significantly higher than in the BC samples (Fig 2.10a; confirmed with Kruskal-Wallis for OTU richness and Shannon Index; \(p\)-value=2e-4 and 4e-6, respectively). The microbiomes of the BC and AK oyster samples were also significantly different when visualized on an nMDS plot (not shown, stress=0.0752) and by PCoA (Fig 2.8b); BC spat had higher sample-to-sample dissimilarity in beta-diversity as shown by wider distribution of plots (Fig 2.8b), whereas beta-diversity of AK juvenile oyster samples were similar to each other (as shown by closer ordination plots to centroid in Fig 2.8b).

![Figure 2.8. Comparison of the microbiomes from AK juvenile oysters and BC spat samples.](image)

(a) Alpha diversity measured with OTU richness, Shannon Index and Pielou's evenness with pair-wise ANOVA; significance values are indicated with asterisks (*\(p\)-value<0.05, **\(p\)-value<0.01, ***\(p\)-value<0.001). The OTU richness and Shannon Index show that the richness, diversity and evenness of the AK oyster microbiome are significantly higher than for the BC spat microbiome. (b) Beta diversity dispersion of the AK and the BC oysters by measuring Bray-Curtis dissimilarity, plotted using Principal Coordinate Analysis (PCoA). An ANOVA-like test was performed with 1000 permutations to measure the homogeneity of the microbiome within each of the AK and BC samples (*\(p\)-value=1e-02). The AK and BC samples have dissimilar microbial composition. The plot shows that the microbiome of the BC samples is more variable compared to the AK samples. Ellipses indicate the 95% confidence interval of the standard error of the ordination drawn around centroids of AK and BC samples.
To determine the components of the microbiome that contribute to differences in beta-diversity across Alaska and BC samples, LCBD analysis was repeated on the combined BC and AK oyster samples (Fig 2.9a). The BC spat microbiome samples were largely composed of bacteria in the phyla *Proteobacteria* and *Bacteroidetes*, with the families *Nitrosomonadaceae*, *Rhodobacteraceae* and *Flavobacteriaceae* being dominant (Fig 2.9b). The beta diversity of the AK oyster microbiomes was largely shaped by the higher proportion of bacteria in the phyla *Planctomycetes* and *Verrucomicrobia* (Fig 2.9a), within the families *Pirellulaceae* and *Rubritaleaceae* (Fig 2.9b).

The phyla and families dominating the AK samples in the LCBD test were also identified by the core microbiome analysis. Operational taxonomic units assigned to *Vibrio* sp. occurred in all AK samples at a detection threshold >190. With a lower threshold of <50, *Bacteroidetes*
(Flavobacteriaceae) and Proteobacteria (Illumatobacteraceae and Helicobacteraceae) were also identified (data not shown). To identify common OTUs found in all samples, the core microbiome analysis was repeated on the combined AK and BC samples. Across all samples, using a detection threshold of 14, OTUs assigned to the genus Sulfitobacter (family Rhodobacteraceae) were present in 98% of the samples, while the genera Loktanella and Litoreibacter (family Rhodobacteraceae) were detected in 90% of the samples. The detection threshold of 14 was chosen because it was the maximum abundance value that identified common OTUs in all oyster samples examined.

2.3.5 The microbial communities were more similar between BC spat and tank water, than between AK juvenile oysters and the FLUPSY water

To estimate the community similarity between the oyster samples and their surrounding water, alpha and the beta diversities were calculated across all the oyster and water samples. No significant (p=0.314 for BC; p=0.298 for AK samples) difference in alpha diversity was observed when the oyster samples were compared with the corresponding water samples, except for the BC tank water, in which the richness was higher than for the BC spat samples (Fig 2.10a). The alpha diversity (richness and Shannon) of the AK juvenile oysters were significantly higher (p=1.17e-4) than for the BC spat. No difference in alpha diversity was observed between the FLUPSY and BC tank-water samples (p=0.355) (Fig 2.10a). When the beta diversity was calculated based on Bray-Curtis dissimilarity, comparing the two groups of oyster and water samples (stress=0.0859), the degree of similarity between the BC oysters and the tank water were higher than that of the AK oysters and the FLUPSY water samples (Fig 2.10b).
LCBD analysis was also conducted between the AK juvenile oysters and the FLUPSY samples to see which taxa were contributing to the dissimilar microbiome structures (Appendix B). The AK juvenile oysters have a higher proportion of OTUs from the phyla *Planctomycetes* (dominated by the family *Pirellulaceae*) and *Verrucomicrobia*; whereas, the FLUPSY water had a higher proportion of *Proteobacteria* (dominated by several families), *Bacteroidetes* (dominated by *Flavobacteriaceae*) and *Thaumarchaeota* (dominated by *Nitrosopumilaceae*) (Fig 2.11).
When the relative abundances of OTUs from different phyla was compared between the AK FLUPSY and BC tank-water samples, the BC tank-water samples had much fewer OTUs assigned to Archaea, while the relative abundances of *Planctomycetes* and *Rhodobacteraceae* (*Proteobacteria*) were similar (Appendix C).

![Figure 2.11. Relative abundance of OTUs by phyla in AK juvenile oysters and FLUPSY samples. OTU abundance was normalized and restricted to the 40 most abundant. Each bar represents a phylum (horizontal axis), and is coloured by family-level relative abundance. The oysters were dominated by the phyla *Planctomycetes* (family *Pirellulaceae*) and to a lesser extent *Verrucomicrobia* (family *Rubritaleaceae*) and *Proteobacteria* (family *Vibrionaceae*). The water samples were also dominated by a diverse array of *Proteobacteria*, although other phyla, including *Thaumarchaeota* and *Bacteroidetes*, which were much less prevalent in the oysters.](image-url)
2.4 Discussion

2.4.1 Microbial community structure between the mortality and the control BC cohorts were similar

Successful cultivation of Pacific oysters in nurseries highly depends on the amount of spat or seed stock the growers acquire from hatcheries. Global mass-mortality events have caused spat shortages, limiting nursery production and destabilizing aquaculture infrastructure.

To identify if potential pathogens or microbial dysbiosis may be associated with mortality events, microbiomes from cohorts of farmed spat that either did or did not experience mortality were compared. There were no statistically significant (p>0.05) differences in the microbial community composition or diversity between the mortality and the control cohorts, but there was a higher relative abundance of *Flavobacteriaceae* in the mortality cohorts. An indicator species analysis revealed that OTUs assigned to the family *Alteromonadaceae* were associated with mortality events; whereas, *Vibrio* species such as *V. coralliilyticus* and *V. tubiashii*, which are potentially pathogenic to *C. gigas* larvae and adults\(^4,39,130\), were not. Thus, analysis of the microbiome of oyster spat indicates that *Vibrio* spp. were not associated with mortality, but that bacteria in the families *Flavobacteriaceae*, *Alteromonadaceae*, the genus *Roseovarius* may be.

The family *Flavobacteriaceae* which was observed in mortality cohorts, is the largest family in the phylum *Bacteroidetes*, and includes aerobic, common marine microbes, as well as pathogenic\(^131\), opportunistic and commensal strains\(^132\), making it difficult to assign a functional association without experiments on isolates. Members of the *Bacteroidetes*, to which the family *Flavobacteriaceae* belongs, and *Proteobacteria* are common phyla in Pacific oysters\(^5\); thus, the presence of *Flavobacteriaceae* is not strong evidence that these OTUs belong to pathogens of oysters. Similarly, members of the *Alteromonadaceae* occur in nutrient-rich seawater, marine
invertebrates and sediments, and display extensive carbon degrading properties\textsuperscript{133}; hence, they may have been opportunistic associates of the dying spat or decaying food, and not causative agents of mortality. Bacteria in the species \textit{Roseovarius crassostrea} have been associated with juvenile oyster disease (JOD) in Eastern oysters (\textit{C. virginica}) from the Atlantic Ocean\textsuperscript{134,135}. However, bacteria belonging to \textit{R. crassostrea} have not been known to infect other bivalve species nor been reported in Canada (DFO Report). Although, my study identified specific OTUs from bacteria associated with spat mortality, it is not possible to infer that these were causative agents of disease.

\textbf{2.4.2 Changes in the microbiome during early spat development}

The thesis is the first to report on short-term changes in the microbiome of early developing oysters. The effect of age on the microbiome of hosts has been extensively studied in medicine\textsuperscript{21} and ecology\textsuperscript{17}, and changes have also been observed in several species of oysters, including \textit{C. gigas}, \textit{C. cortesiensis} and \textit{C. sikamea}\textsuperscript{5,6}. However, in these studies age was based on major life stages (i.e. larvae, spat vs. adult), and not on the microbial dynamics within a life stage. The time after metamorphosis is particularly relevant, as developing oysters are susceptible to diseases, observed in other oyster species\textsuperscript{136,137}. The shift in microbial community composition in developing oyster spat sampled on weekly basis has not been reported nor inferred as a “maturing” microbiome. Specifically, during the first two weeks following metamorphosis, there was a gradual shift in the abundance of bacteria in the phyla \textit{Proteobacteria} and \textit{Bacteroidetes}. A similar change in the relative abundances of bacteria in these phyla has been observed in other studies although the comparison was between one life stage to another (spat vs. adult \textit{C. gigas})\textsuperscript{5,6,58}. 
There was no similar change in microbial composition in the AK juvenile oysters, although there was a gradual increase in OTUs assigned to the *Planctomycetes*. As well, bacteria in the phylum *Firmicutes* and the genus *Vibrio* were core members of the AK juvenile oyster microbiome, and are common in Pacific oysters; *Firmicutes* have been exclusively associated with adult oysters\(^5\). The differences in community dynamics between the spat and juvenile oysters highlights the need to understand changes in the microbiome of developing oysters.

The youngest spat showed more dissimilarity in microbial structure compared to average of total sample microbial structure. For example, Local Contribution to Beta Diversity (LCBD) analysis on BC spat organized by age groups showed that all but one samples belonging to the youngest age-group (0-5 d) had high LCBD values indicating microbial structure from each sample was markedly different from total average beta-diversity (Fig 2.7a). The differences of beta-diversity of youngest BC spat to the average beta-diversity was also supported in the beta-dispersion test as the range of ordination points were more widely distributed away from the centroid (Fig 2.10b). It can be speculated that the marked difference of beta-diversity of each sample within the youngest age-group may indicate 1) a stochastic microbial structure of diseased spat\(^1^4\); b) a snap-shot of rapid microbiome changes immediately after metamorphosis as it was observed in other aquatic species\(^1^3^8\). However, without data from wild spat immediately after metamorphosis, the effect of metamorphosis on causing stochastic or markedly different structure from average beta-diversity cannot be accurately determined. In addition to the changes in the relative abundances of sequences assigned to *Proteobacteria* and *Bacteroidetes*, more reads were assigned to the proposed superphylum *Patescibacteria*, and fewer to the phylum *Chloroflexi* in the earliest age-group. The presence of bacteria belonging to *Patescibacteria* is significant, as they have not been reported in *C. gigas* spat, and are postulated to be from clades
that include known ectosymbionts\textsuperscript{139,140} and parasites\textsuperscript{141}. In contrast, \textit{Chloroflexi}, which encompass a metabolically diverse group of bacteria, have been reported in the gut contents of other adult oyster species\textsuperscript{7,128}, suggesting that they may become a feature in the microbiomes of oysters as they age.

Mortality of the spat always occurred in the first two weeks after metamorphosis during the time when the composition of the microbiome was changing markedly. Evidence for this is the generally high relative LCBD values ($\geq 0.040$) associated with the youngest oysters, indicating low taxonomic overlap with the microbiome of older spat; thus, the early stage of spat development may be a period when the composition of the microbiome is particularly unstable, resulting in a higher potential for dysbiosis\textsuperscript{123}. The relationship between an unstable microbiome and disease susceptibility has been proposed as an ‘Anna Karenina principle’ (AKP), and emphasizes the need to investigate microbiome stability, rather than just focusing on diseased versus healthy microbiome patterns\textsuperscript{14}. These results provide the first glimpse into the dramatic changes in the microbiome of post-metamorphic oysters, and hint at the need to understand how these rapid changes may be related to the health of developing oysters and disease susceptibility. To further test if the spat were diseased, it would be necessary to quantify the stochasticity of the spat microbiome following infection with known pathogens to differentiate disease-related from age-related shifts in the microbiome.

Aside from a phylum \textit{Proteobacteria}, sequences assigned to a phylum \textit{Planctomycetes} showed similar pattern in older BC spat and the AK oysters even though the sampling time, production stages and farm sites were different. The gradual increase in the proportion of taxa within the \textit{Proteobacteria} as the spat aged was followed by a transient increase in \textit{Planctomycetes} in older spat (Fig 2.6). \textit{Planctomycetes} were also the most abundant phylum in
the AK juvenile oysters (Fig 2.9b). The trend of increasing *Planctomycetes* in aging BC spat and their ubiquity in the AK juvenile oysters, suggests that bacteria in this phylum are key members of the microbiome of growing oysters, and is consistent with the idea of a maturation process of the oyster-spat microbiome\(^5\). *Planctomycetes* were also found in gill tissues\(^{54,66}\) and homogenate\(^{59}\) of Pacific oysters, although they were relatively more abundant in gill tissue\(^{5,59}\). Hence, bacteria in the *Planctomycetes* may be specifically associated with gill tissue as it may get concentrated in the tissues during filtration. The differential abundance observed in previous studies may be due to environmental factors such as temperature, salinity, or oxygen level\(^{142}\) which may play role in a *Planctomycetes* dynamics in surrounding water\(^{9,59}\).

### 2.4.2 Bacteria in BC and AK oysters may include sulfur and ammonia oxidizers

Comparing the microbiomes of AK juvenile oysters and BC spat revealed that the genus *Sulfitobacter* (family *Rhodobacteraceae*, phylum *Proteobacteria*) occurred in 98% of the samples. Bacteria belonging to *Sulfitobacter* oxidize inorganic sulfur, sulfite and thiosulfate, and have associated activities such as chemical defense in sponges and marine oil degradation\(^{10,143}\). The filter feeding nature of the Pacific oysters, and evidence of sulfur oxidation in bivalve reefs, suggests that *Sulfitobacter* may be functionally ubiquitous to filter-feeding bivalve species\(^{43}\). Moreover, bacteria in the genus *Sulfitobacter* produce secondary metabolites with a range of defense functions with antiviral, antibacterial and antitumor effects\(^{143}\). Their functional relationship with Pacific oysters remains to be explored using metabolomics and chemical assays.

A closer look at changes in the microbiome of oysters as they age showed that members of the phylum *Planctomycetes* were common in older BC spat (family *Planctomycetaceae*) and AK juvenile oysters (family *Pirellulaceae*). Members of the phylum *Planctomycetes* include
major nitrogen-fixers$^{12,144}$, and have been associated with deep-sea octocorals ($Paramuricea placomus$)$^{145}$ and kelps ($Macrocystis pyrifera$)$^{144}$. Evidence of their potential importance can be seen in suspended oyster farms of Sydney Rock oysters ($Saccostrea glomerata$) in which nitrification of nitrite ($NO_2^-$) to nitrate ($NO_3^-$) and inorganic nitrogen retention were observed$^{42}$. Given the filter-feeding nature of bivalves, it is possible that these taxa are common in filter-feeding marine organisms and may be important in biogeochemical cycles$^{12,144}$.

2.5 Conclusion

In this study, the microbiome in mortality and control cohorts of BC farmed oyster spat were compared. While no significant difference in community composition was observed in spat experiencing mortality, there were changes in the composition of the microbiome as the spat aged. These rapid changes in microbial composition immediately after metamorphosis and setting reflect a period of microbiome instability that could make them susceptible to mortality. Alternatively, the rapid change could indicate a diseased state of spat in which the symptoms were appearing as either stochastic microbiome structure or mortality events.

Future investigation of the functional association of the core microbial taxa and different microbial structures associated with developing spat would benefit from additional sampling efforts from broader range of developmental period, from broodstock, eggs, larvae, spat, and juveniles in weekly manner. If possible, similar sampling regimes should be replicated with wild Pacific oysters or duplicated in another farm site. In addition to sequencing 16S rRNA amplicons, thorough examination of functional gene expression throughout oyster development should be analyzed to determine roles of associated microbiome. Comparing these data to oysters that experienced mortality events can better pinpoint if the underlying cause is immunity, metabolism, or developmental related. Furthermore, finer timeseries comparison of microbiome
data throughout different life stages of oysters can provide how transition between life stages affect stability of microbiome, hence providing more information about disease- or dysbiosis-susceptible period.

Taxa associated with nitrogen fixation and sulfur oxidation that occurred in the AK and BC oysters suggest a functional role for these groups of microbes. However, to verify if the functional relationship of these microbes is symbiotic or beneficial to the animals’ health, requires laboratory experiments involving isolation of associated bacteria, antibiotic administration followed by mortality rescue (if any) by introducing the isolated bacteria, and measurements of organic sulfur, inorganic nitrogen and other metabolites to see if it affects fitness of the animals. Furthermore, changes in gene expression or protein level can be accessed using transcriptomics or metabolomics to examine involvement of the associated taxa in oyster metabolism.
Chapter 3: Conclusion

3.1 Summary of results

Previous microbiome studies of Pacific oysters consisted of disparate microbiome data demonstrating developmental changes during hatchery production. For instance, many studies focused on characterizing the pathobiome of Pacific oysters that were experiencing mortality events or were under abiotic stress (e.g. temperature\textsuperscript{9,59}) from diverse geographic locations, sampling time, life stages (mostly adults), and growing conditions (e.g. farmed\textsuperscript{7}, wild\textsuperscript{4,61}, or lab\textsuperscript{5}). Additionally, the choice of tissues, sample processing methods, DNA extraction protocols, target 16S rRNA gene region, and sequencing platform differed among the studies. Despite the large variation in approaches to oyster microbiome studies, common phyla associated with specific life stages or different abiotic stress were repeatedly observed, although some findings were conflicting (Table 1.1).

In the current study, high-throughput sequencing was used to examine the microbiomes of hatchery-reared Pacific-oyster (\textit{C. gigas}) spat from British Columbia (BC), and juveniles from Alaska (AK). There were two parts to the study. In the first part, the microbiomes of cohorts of BC spat that experienced mass mortality were compared with those that did not. In the second part, the microbiomes between the BC spat and the AK juveniles were compared with the goal of finding shared taxa between oysters of different ages, from distant locations that were growing under very different conditions.

3.1.1 Microbiome shift of the early developing Pacific oysters

The Pacific oyster, \textit{Crassostrea gigas} is one of the most cultivated shellfish in aquaculture worldwide, meeting growing global seafood demand\textsuperscript{1,37}. With mass mortality events in hatcheries causing seed-stock shortages, the industry faces challenges in acquiring seed (spat)
and maintaining nursery production. To investigate potential causes of mortality, the microbiomes of spat farmed in BC that had experienced, or had not experienced mortality were characterized by sequencing the V4-V5 hypervariable regions of the 16S rRNA gene.

To investigate changes in microbiome composition, rapidly growing, just-set spat, were sampled weekly from day 0 to a maximum of 74 days. Microbial composition changed; for example, there was a gradual decrease in the relative proportion of sequences assigned to the phylum *Bacteroidetes*, while those assigned to *Proteobacteria*, increased. Other studies have reported *Bacteroidetes* being more abundant in oyster spat; whereas, *Proteobacteria* were in higher relative abundance in adults. However, the current study documents this shift over a period of weeks in growing spat. There were other changes as well, with the phylum *Chloroflexi* gradually increasing in relative abundance, *Patescibacteria* decreasing, and *Planctomycetes* peaking between 6 and 23 days, as the spat aged. The results of BC spat microbiome data was different from AK oysters which did not show significant (Kruskal-Wallis; p>0.05) differences in the composition of their microbiome across age classes although a gradual increase in the relative abundance of *Planctomycetes* and a decrease in *Verrucomicrobia* were observed (Fig2.9b).

The change in the composition of the microbiome in BC spat as they age, and its relative stability in older spat and AK juvenile oysters, suggests a developmental progression followed by stabilization in the microbiome as the spat age. Although this was generally true for the nine cohorts of spat followed in this study, it remains to be seen if this is a common feature of spat grown at other locations, and under different conditions. Furthermore, the composition of the microbiome of young spat was more variable among cohorts than in older spat. It has been
reported that there was greater variability in the microbiome of moribund adult oysters than in healthy oysters\textsuperscript{4}, suggesting variability can be related to disease.

### 3.1.2 Hypotheses findings and integration

This study was designed to test the following two hypotheses: First, oyster spat from hatcheries in BC, and juvenile oysters in AK, share common taxa in their microbiomes. Second, specific microbial taxa are associated with mortality events in farmed oyster spat. The results of the study in the context of these hypotheses are discussed below.

The composition over time of the microbiomes in BC spat and AK juvenile oysters was determined based on OTU prevalence and abundance. Alaskan juvenile oysters had higher relative abundances of OTUs in the phyla *Planctomycetes* and *Verrucomicrobia*; whereas, BC spat were dominated by OTUs assigned to the phyla *Proteobacteria*, *Bacteroidetes* and *Planctomycetes*. However, the AK juvenile oysters and BC spat shared OTUs assigned to the genus *Sulfitobacter* (*Proteobacteria*), and to the families *Planctomycetaceae* and *Pirellulaceae* (*Planctomycetes*). The latter two families comprise sulfur-oxidizing and nitrogen-fixing bacteria, respectively\textsuperscript{12,13,143}. Other taxa, such as the genus *Loktanella* (family *Rhodobacteraceae*), the family *Saprospiraceae* (phylum *Bacteroidetes*) and the genus *Lentilitoribacter* (family *Rhizobiaceae*) were in ~80\% of the samples, but in low relative abundance; hence they may be common commensal species although their functional roles are to be determined. To determine if the co-occurring taxa assigned from shared OTUs down to the family or genus level in this study are widely occurring in Pacific oysters and other bivalves would require sampling a wide range of other shellfish, sympatric species, and Pacific oysters of different ages, and from different farm sites, natural habitats and times.
It has been suggested that the external microbiota of the environment and the development of the filtration organ determines the microbiome dynamics as the spat develops\textsuperscript{8,26}; however, the analysis conducted here did not capture the effect of water microbiome on developing spat since the beta-diversity of water from different tanks and time was similar to each other (Fig 2.12b). It is possible that the shared OTUs between the AK juvenile oysters and BC spat stemmed from filter feeding where the oysters selectively retain microbes or certain microbes accumulating in oyster tissues as habitat niche; however, the effects of the common taxa found in AK and BC oysters on the oyster survival, particularly for the developing spat remains unknown.

To test the second hypothesis that specific taxa or a taxon are associated with mortality, cohorts of spat that experienced mortality were divided into bins representing before, during and after peak mortality. In turn, each mortality cohort was paired with a control cohort of oysters that was set at about the same time, but which did not experience mortality. There was no significant difference (Kruskal-Wallis pairwise test; p>0.05) in microbial community structure between the mortality and the control groups. However, indicator species analysis revealed that OTUs in the order Alteromonadales (class Gammaproteobacteria) were associated (p<0.05) with all the mortality events, while OTUs in the order Sphingobacteriales (class Bacteroidia) were only associated with the before-mortality group. Members of the order Alteromonadales have been reported to have extensive carbon degrading properties, are found in wide range of marine environments including nutrient-rich areas, sea ice, sediments, and marine invertebrates\textsuperscript{147}. The order contains all the known psychrophilic (cold-loving) and most barophilic (pressure-loving) bacteria, suggesting that it may originate from upwelled deep water\textsuperscript{147}. However, demonstrating
that bacteria in the *Alteromonadales* causes mortality would require infection assays with isolates.

### 3.2 Interpreting shifts in the microbiome and mortality in spat

The current study demonstrated clear shifts in the microbiome of spat reared in BC hatcheries, regardless of mortality events. The age-related microbiome shifts in oysters have been reported as changes in the relative abundances of OTUs from phyla in other oyster microbiome studies\(^4\)-\(^7\),\(^9\). The microbiome within the youngest age-group of spat also showed higher dissimilarity in beta-diversity compared to the total average beta-diversity, indicating considerable microbiome instability during early developing spat. Additionally, compared to the AK juvenile oysters, the beta diversity of the spat also showed higher dissimilarity among each other, indicating more stable microbiome may be established in juvenile oysters. The shifts in the composition of the microbiome were greatest in the youngest oysters, such that the beta-diversity was significantly different (<0.05) among age-groups of BC spat, and more heterogeneous than in the AK juvenile oysters. Although these shifts are associated with the age of the spat, the cause of the shifts cannot be inferred from the data that were collected. For cohorts of spat that experienced mortality events, peak mortality always occurred within the first two weeks following metamorphosis. It is possible that the instability and rapid change in the microbiome community structure that occurred within the first few days after metamorphosis provided an opportunity for opportunistic pathogens to invade the microbiome leading to mortality; however, the OTU data indicates that it would have to be a relatively small component of the overall community, as the microbial community structure was not significantly different between cohorts that experienced mortality, and those that did not.
To disentangle potential effects of farm handling stress, opportunistic pathogens, environmental variables and developmental changes on mortality events in spat is difficult given that all of these factors may play a role. The fact that mortality occurs rapidly and is restricted to specific cohorts strongly hints a pathogen. However, even if a pathogen is involved, the cause of mortality could be multifactorial and the result of multiple stressors.

The current study used amplicon sequencing approach targeting 16S rRNA gene which would have only allowed detecting prokaryotic pathogens. It is possible that the pathogen was of eukaryotic or viral origin, and further screening should be repeated on the examined BC spat targeting appropriate eukaryotic genes\textsuperscript{148} or deploy alternative sequencing method (e.g. RNA-seq\textsuperscript{149}). In addition, to ensure pathogenic activities, global gene expression showing antiviral or antibacterial activities of oyster spat should be examined. Furthermore, correlation of highly dissimilar microbiome structures among developing oysters and animal survival should be analyzed in addition to characterization composition of microbiome structures\textsuperscript{14}.

### 3.3 Potential abiotic and biotic factors from seawater associated with mortality events

The cause of the mortality events in spat may involve the seawater. The incoming water used in the nursery tank was sand-filtered, 5-\(\mu\)m filtered, and adjusted to the optimal temperature, pH, and alkalinity for spat growth. Therefore, all the spat cohorts were grown under similar environmental conditions. There was no correlation between mortality events and conditions in the pre-adjusted water data (Appendix A). Despite the similar water quality among cohorts, the alpha diversity in the seawater was significantly different (p<0.05). The differences might be the result of changes in nutrient levels, aragonite concentration, salinity, food quality or other factors that could change with time and influence microbial composition of the seawater\textsuperscript{150}. 
The potential cascading effect of the seawater microbiome on the spat could also affect the broodstock, which could also affect the larvae and spat through the vertical transmission of symbionts as has been observed in other marine animals such as corals\textsuperscript{75} and salmonids\textsuperscript{29}. The hatchery operators in BC acquired naturally spawning adult oysters and artificially fertilized the eggs by stripping the gonads. Although Pacific oysters are known to be resilient against wide ranges of temperature, salinity, and pH, the integrity of the microbiome may be less resilient leading to loss of essential microbes during various environmental conditions (e.g. ocean acidification or warming) and strip-spawning practice\textsuperscript{83,102}. Therefore, adult oysters with stress-exposed microbiota may lack microbes that are essential for spat survival or contain microbes that are detrimental to the spat. In addition abiotic stress could cause the microbes to produce metabolites that can trigger physiological responses and affect the spat immune response\textsuperscript{151–153}.

As mentioned previously, another strong possibility is the pathogen could be eukaryotic, viral, or a mobile genetic element, factors that were not detected by targeting 16S rRNA regions. Even if non-bacterial pathogens did not directly cause mortality events, they could be the source of virulent genes, that were horizontally transferred to commensal microbes in the oysters\textsuperscript{154,155}. Targeted amplicon-sequencing of micro-eukaryotes\textsuperscript{148}, and transcriptomic sequencing to detect viral replication are approaches that could be used to further explore the potential role of pathogens\textsuperscript{156,157}.

3.4 Final synthesis and future direction

Rapid development of next-generation sequencing (NGS) has drastically shifted our views on pathological interactions\textsuperscript{22,69}. The new perspective arose from the realization that there are diverse roles of the microbiome, including acting as a multi-faceted ‘exogenous’ organ that can provide a protective immune barrier\textsuperscript{104}, metabolic functions\textsuperscript{41}, and be a buffer for abiotic
stress and serve as a genetic reservoir\textsuperscript{70,158}. The compromised integrity of the microbiome (i.e. dysbiosis) can also be fatal to the host by exposing it to opportunistic pathogens or depriving it of essential metabolites. Such complex interactions may explain the mortality events occurred in BC hatcheries in which a single pathogenic agent has not been identified.

Thus, the increase in mortality events in farmed Pacific oyster spat could be dysbiosis caused by environmental changes such as ocean acidification\textsuperscript{67} and warming; thereby, exposing the spat to a new or opportunistic pathogen, or triggering commensal microbes to become virulent\textsuperscript{6,159}. Instead of dramatic difference in microbial community structure between cohorts that experienced mortality or did not, the changes in microbiota were modest, and were largely independent of mortality events. The shift was predominant in young spat, and mortality always occurred within the first two weeks following metamorphosis. Stabilization of the microbiome in young spat may be critical in establishing resilience against abiotic or biotic stresses\textsuperscript{14}.

\textit{Osterid Herpes-Virus (OsHV)} and \textit{Vibrio} species have been implicated in many mortality events in Pacific oysters; however, it is not clear if these pathogens target particular life stages\textsuperscript{33,87}. The mortality events observed in spat may potentially be novel, since the \textit{OsHV} has not been reported in BC, and the sequence data showed no evidence that \textit{Vibrio} spp. were associated with mortality. Regardless of the mortality events, the rapid shift of the microbiome within the first 2-3 weeks of spat setting reflects how the microbiome may go through its own ‘metamorphosis’ as the planktonic larva transition into a sessile stage. The effect of the shifting microbiome on the fitness of spat should be further examined by comparing the microbiome from each life stage of \textit{C. gigas}, from broodstock to adult oysters. Furthermore, the effect of biotic (microbes of seawater) and abiotic (water chemistry and farming procedures) factors on the microbiome should be examined, based on carefully designed laboratory experiments and
various -omics sequencing technologies. Ultimately, the effect of the microbiome on spat fitness may be taxon-based (a few key species) or community based (a change in overall composition).

3.4 Steps to applying microbiome studies to shellfish aquaculture

The application of microbiome research to commercial shellfish growing is not new. Commercially available microbiome-based aquaculture products such as Pond Plus (Novozymes, Bagsværd) and Toyocerin® (Rubinum, Barcelona) are geared towards enhancing water quality by adding bacterial assemblages that reduce ammonia and nitrite and use select microbes as feed additives to strengthen intestinal mucosa\textsuperscript{160}. The taxonomic and functional complexity of the microbiome and how it changes with life stage, abiotic stresses such as ocean acidification and warming temperatures, and biotic stresses such as exposure to novel microbes, makes it challenging to predict how probiotic treatments will interact with the developing spat. Future microbiome studies should use experimental approaches to test the effects of changing environmental conditions on \textit{C. gigas} reproduction and fitness of the offspring, and what functional roles the microbes play. Meaningful association of microbes with farmed oysters requires characterizing both the taxonomy and the functional roles of the microbes associated with oyster spat. This could be approached by looking at expressed genes and metabolites that can affect pathogenicity\textsuperscript{161}.

3.5 Impact and implications of the current study

The results from this thesis showed dynamic shifts in the microbiome of early developing Pacific oyster spat grown in a hatchery. When compared to juvenile oysters, the composition among spat microbiomes were more dissimilar among each other; whereas, the juvenile oysters harboured a similar within-group microbiome. Although efforts have been made to characterize temporal dynamics of Pacific oyster microbiomes in previous studies, these studies often
compared major life stages, such as larvae versus adults and neglected transitional changes in the microbiomes of within life-stage spat, which is the stage that hatcheries largely cultivate. Based on these findings, I strongly recommend that future studies focus on effects of oyster larvae metamorphosis in microbiome dynamics, and the time it takes for the dissimilarity in microbiome structure among spat to transition to a more stable microbial community. It may lead to identifying factors (e.g. gastrointestinal organ maturation) that causes dynamic shifts in early spat growth, furthermore providing direction in finding cause(s) of mortality.

Although no mortality-related microbiome “signature” was found in this study, a few indicator species (some at the genus level), including the family Alteromonadaceae and genus Roseovarius were associated with mortality cohorts. Future studies can try to isolate these indicator species as some are pathogenic and determine their infectivity. Further transcriptomic or metabolomic analysis may reveal if these indicator species can affect host immune systems.

Mass mortality events observed in BC spat cohorts are most likely combination of multi-factors and tackling each factor will be time-consuming and impractical to help shellfish growers. With careful interpretations, microbiome of organisms can show influence from the hosts and environments (including pathogens) as both symptoms or aetiological causes. Further research that incorporates various factors into examining the microbiome in developing Pacific oysters in aquaculture will help to identify mortality causes and contribute to higher production of Pacific oyster seeds.
References


67. Wang, Q. et al. Effects of ocean acidification on immune responses of the Pacific oyster


Appendices

Appendix A Canonical correspondence analysis (CCA) of microbial taxa and constrained environmental variables in the BC nursery tanks.

The significance of the correlation (Pr>r) or p-values were measured based on random permutations of the fitted vectors (p-value cutoff <0.05). When the vectors were specified in a constrained model on canonical correspondence analysis (CCA), there was a significant correlation with age (‘Days since set’), but not with environmental parameters (p-value=0.043). The samples are coloured based on when the mortality occurred; No=samples from no mortality cohorts (n=23); Yes-after=sampled after the mortality peak (n=11); Yes-before=sampled before the mortality peak (n=3); Yes-during=sampled during mortality peak (n=2).
Appendix B Analysis of Local Contribution of Beta-diversity (LCBD) comparing AK juvenile oysters and FLUPSY water.

The LCBD bar graphs showing relative proportion of the top 21 taxa that contributed to the difference in microbial composition between the FLUPSY water samples and the AK juvenile oyster. The degree of compositional uniqueness is represented by the size of the black spheres above the sample names. The oysters have higher proportion of *Planctomycetes* and *Verrucomicrobia*; whereas, the FLUPSY water samples have a higher proportion of *Proteobacteria* and Archaea.
Appendix C Comparison of relative abundance in different phyla between AK FLUPSY water and BC tank water filtrates.

A bar graph generated by normalizing reads by sequence depth, showing different relative abundances of each phylum in AK FLUPSY water and BC Tank-water. Each bar represents a phylum, and is filled with the relative abundance of its corresponding family level. Both water samples had the highest relative abundance of *Proteobacteria*, and similar abundances of *Bacteroidetes* and *Planctomycetes*. However, the AK FLUPSY water samples showed higher abundances of *Thaumarchaeota*, *Euryarchaeota*, and *Actinobacteria*, which were present in low levels in BC Tank water samples. More diverse family levels were present in *Proteobacteria* found in AK FLUPSY water samples while higher abundance in *Verrucomicrobia* was found in BC Tank water.