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The oyster larval microbiome and its manipulation for the improvement of shellfish aquaculture

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Secondary supervisors: Dr Tim Regan, Dr Amanda Warr, Dr Frederico Batista and Dr David Bass.

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Declaration

I declare that this thesis was composed by myself. The research presented was conducted by myself from between September 2020 and December 2024 with the following exceptions:

- Optimisation of universal 16S primers used was carried out by secondary supervisor Dr Frederico Batista. This primer design was used for 16S sequencing in chapters two, three and four.
- Sample collection for chapter two was carried out by Dr Erica Chapman (St Abbs Marine station) and Professor Bill Sanderson (Heriot-Watt University).
- DNA extractions for chapter four were carried out by supervisor Dr Frederico Batista, who also organised the shipment of these samples to the Roslin Institute.
- Mortality count data for chapter five was collected in part by primary supervisor Dr Tim Bean and Kallen Sullivan of the Roslin Institute's Aquaculture team.

This thesis has not been submitted, in whole or in part, in any previous application for a higher degree. All sources of information have been acknowledged in the text.

Hannah Farley

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List of abbreviations

AMR	Antibiotic resistant bacteria
ASV	Amplicon associated variant
CCAP	Culture Collection of Algae and Protozoa
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic acid
Dpf	Days post fertilisation
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FT	Flow through
Hpf	Hours post fertilisation
HTS	High throughput sequencing
ID	Identification
IMS	Institute of Marine Science
LROFC	Loch Ryan Oyster Fishery Co. Ltd
LSK	Ligation sequencing
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
MR	Metabolic rate
MSX	Multinucleated Sphere Unknown
NMDS	Non-metric multidimensional scaling
ONT	Oxford Nanopore Technologies
OsHV-1	Ostreid herpesvirus

OSPAR	Oslo and Paris conventions
OTU	Organised Taxonomic Unit
pCO_2	Partial Carbon Dioxide
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
pH	Potential of Hydrogen
POM	Particulate organic matter
POMS	Pacific Oyster Mortality Syndrome
Ppt	Parts per thousand
rRNA	Ribosomal RNA
RS	Recirculating system
SD	Standard deviation
TDA	Tropodithietic Acid
UK	United Kingdom
UV	Ultraviolet
V1	Hypervariable region 1
V4	Hypervariable region 4
V6	Hypervariable region 6
WGS	Whole genome sequencing
ZM	Zobell Marine

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Thesis abstract:

Aquaculture is the practice of farming aquatic species and contributes significantly to global seafood production. Aquaculture production has increased dramatically since the 1950's, and with this, so has the production of molluscs. Oysters make up the most commonly produced group of molluscs by quantity, being produced for both food sustainability and ecological restoration goals. Importantly, the security and expansion of oyster farming is reliant upon reliable production of good quality seedstock from hatcheries. The production of such seedstock is, however, greatly hindered by large-scale disease mortality events that often occur during the larval stages of the oyster lifecycle. The causes of these disease mortality events are known to be often caused by dysbiosis of the larval microbiome (the collection of microorganisms including bacteria, viruses and fungi that live within the larvae and their environment). It is also is well accepted that the microbiome plays a key role in maintaining larval health. Research into the bacteria that reside within oyster larvae and their environments is therefore vital to understand larval health and improve the ways in which they are farmed. This research is also of benefit to aquaculture generally with methods of improving oyster aquaculture being possibly applicable to other key bivalve species such as mussels, scallops or clams. Within a broader context still, the improvement of oyster aquaculture is beneficial to restoration efforts, where increased oyster production allows for the improvement of water quality, substrate stabilisation and increased biodiversity in coastal ecosystems.

This thesis aims to improve the current understanding of the larval microbiome and investigate possible methods of microbial manipulation for improved larval heath within the hatchery setting. Chapter two addressed this with an environmental microbiome study of the European flat oyster (*Ostrea edulis*). This was done via the collection of water samples taken throughout a natural spawning event of *O. edulis* at Loch Ryan, UK. It was reported that microbial changes within this spawning event were most closely correlated with date and driven by the differences in abundance of certain bacterial taxa such as those belonging to the Rhodobacteraceae family. Inverse abundance profiles were also observed between bacteria belonging to the Rhodobacteraceae family and *Vibrio* genera. With species of the *Vibrio* genera being known to cause disease mortality events in larvae, this finding allowed

for the formation of a hypothesis that *Sulfitobacter* and *Jannaschia* genera of Rhodobacteraceae may be suitable probiotic candidates for usage within oyster hatcheries. This hypothesis was tested through both bacterial plate assays and *in vivo* challenges in adult oysters throughout chapter five. Although preliminary, these challenges provided promising data. Most importantly, the addition of Rhodobacteraceae to seawater improved oyster survival rate when challenged with *Vibrio aestuarianus*. This finding has possible applications to improving oyster heath and cultivation. Chapter five also discusses future experimental work needed to replicate these findings within larger sample sets.

Similarly, chapter three was carried out with the ultimate aim of improving larval health within the hatchery environment for improved oyster production. This chapter again uses full-length 16S rRNA sequencing to provide baseline bacterial profiles throughout commercial production at two different UK hatcheries, covering species of both *O. edulis* (produced at Portsmouth) and *Crassostrea gigas* (*C. gigas,* produced at Morecambe Bay). The core larval microbiomes from each hatchery were identified and microbial species richness tended to decrease as the larvae developed. Microbial profiles of larvae were also found to be distinct to that of their surrounding water. This broad-scale microbial data also allowed for the microbial implications of hatchery practices to be discussed with water filtration techniques at both locations being successful in reducing bacterial diversity within the water samples. Generally, this chapter improves the current understanding of the larval microbiome and their hatchery environment whilst also fulfilling a knowledge gap by conducting this research throughout commercial production.

Chapter four builds upon previous baseline data by investigating possible methods of microbiome manipulation to improve larval health and negate the need for antibiotic usage within hatcheries. The growth rates of larvae which were reared up to 15-days post fertilisation under different diet and treatment groups were collected. Microbial comparisons were also reported via both culture dependent and independent techniques. Most notably, the microbiomes of larvae grown under two different dietary treatments were found to be distinct from one another, with differences being driven by the abundance of bacterial taxa such as Actinobacteria, Bdellovibrionia and Bacilli.

In summary, this thesis provides vital baseline data about the *O. edulis* and *C. gigas* larval microbiomes throughout a range of different spawning types and locations. This has allowed

for an improved understanding of the larval microbiome which is vital in order to better inform hatchery practices and improve production. Chapters four and five build upon the previous broad-scale data collected in two and three by demonstrating possible methods of targeted microbial manipulation.

Lay Summary:

Oyster farming is practiced all around the world, supporting global economies as well as contributing to sustainable protein restoration and ecological restoration. Importantly, oysters begin their lifecycle as free-swimming larvae which then transform into small immobile animals known as spat before maturing into market size adults. It is the consistent and reliable production of this spat that the oyster industry is so heavily reliant upon. However, spat production is often hindered by large-scale mortality events which take place during the larval stages of the oyster life cycle. It is well understood that specific bacterial species are often the cause of these disease events. Similarly, it is known that the bacteria and other microorganisms present within oyster larvae and their environment play a key role in maintaining their health, with some being beneficial.

This thesis therefore aims to improve the current understanding of the link between the bacteria that reside within oyster larvae/ environments and their health. To do this, we report bacterial profiles throughout oyster larval development events in both natural and farmed settings. During a natural oyster spawning event, changes in bacterial communities were most closely associated with the date of sample collection. This provided important baseline information needed to improve the current understanding of oyster biology. In farmed settings, core bacterial taxa and their changes overtime were also tracked. This allowed for the identification of specific groups of bacteria which may be important for their health and development. Possible methods of manipulating the larval microbiome through diet or temperature control were also investigated in order to limit the need for antibiotic usage within hatcheries.

Ultimately, this thesis collects broad-scale microbiome data from a range of different oyster species and environments. The findings presented underscore the importance of bacteria to larval health and highlight bacterial manipulation as an untapped method of improving oyster production in hatcheries.

Chapter one: Literature review



1.0 Introduction:

1.1 The importance and growth of aquaculture:

The term aquaculture is broadly used to describe the farming of a wide range of aquatic organisms. Aquaculture, in its simplest form, has been practiced for thousands of years, but technological advances in the past half century have enabled its expansion into a widespread global industry. This recent growth of aquaculture often referred to as the 'blue revolution', has seen an increase in global productivity of 8% per year, resulting in an output of 80 million metric tonnes in 2016, with a value of $232 billion USD (Garlock et al, 2020; Kobayashi et al, 2015). However, whilst aquaculture is a global industry, the vast majority (91%) of production occurs in Asia (Houston et al, 2020). The three biggest contributors are China, Indonesia and India respectively (Figure 1); (Houston et al, 2020; Garlock et al, 2020; Humphries et al, 2021). Possible reasons for this uneven production around the world are linked to variations in farming areas, climatic and market factors as well as social and economic differences across nations (Nadarajah and Flaaten 2017). Aquaculture's continued expansion will allow for meeting the global demand of the seafood industry with cultured seafood now being one of the world's most highly traded commodities (Jennings et al, 2016).

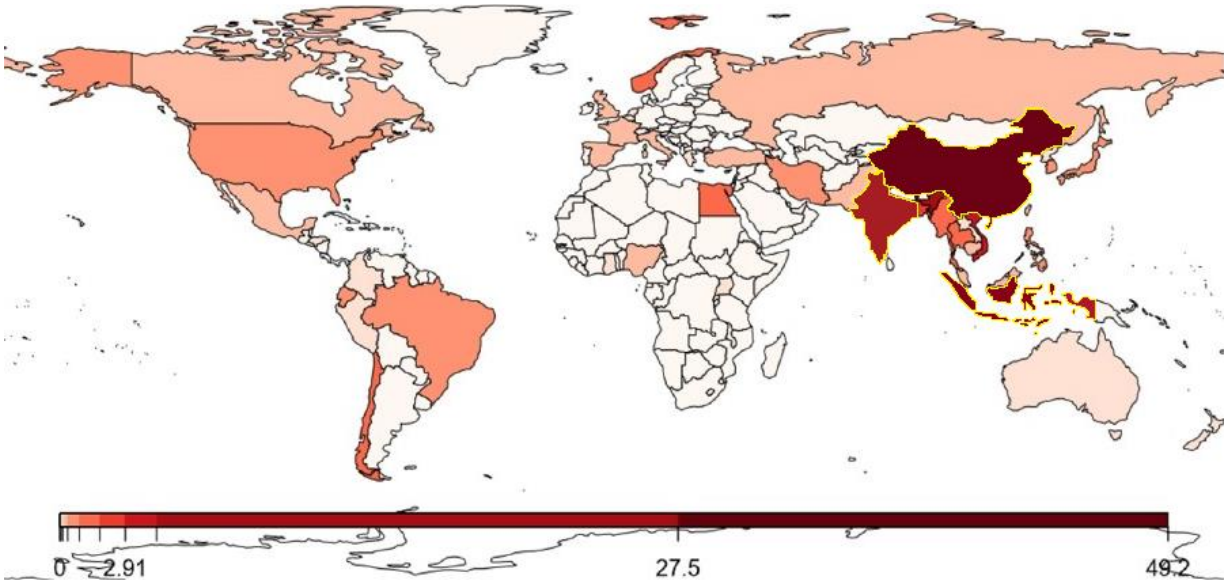


Figure 1: Distribution of aquaculture production across the world in million metric tons. Yellow borders highlight the three highest aquaculture producing countries. Figure adapted by author from Garlock et al, 2020 and FAO, 2018 statistics.

Trends in aquaculture versus capture fishery outputs further demonstrate the growing importance of aquaculture as a source of seafood. For example, whilst production from capture fisheries has remained consistent at approximately 90 million tons per year since the mid 1990's (Kobayashi et al, 2015), aquaculture production shows no signs of becoming stagnant and is now the fastest growing farmed food sector in the world (Houston et al, 2020); (Figure 2). This production is predicted to increase further still with economic models suggesting an output of global fish supply from aquaculture to exceed 180 million tons in 2030 (Kobayashi et al, 2015). In comparison, aquaculture production has already exceeded that of capture fisheries, producing 18.32 million tonnes more in 2019 (Tacon, 2020). Throughout the expansion of aquaculture, the capacity for it to function alongside managed fisheries has been greatly improved. Where wild-capture fisheries collapse due to overexploitation, production from aquaculture is able to fill this gap. Indeed, the total fisheries sector (capture and aquaculture) output has been growing at a rate of 3.2% each year since 1961. This is mainly attributed to the growth of aquaculture which is now able to support capture fisheries (Pauly and Zeller, 2017).

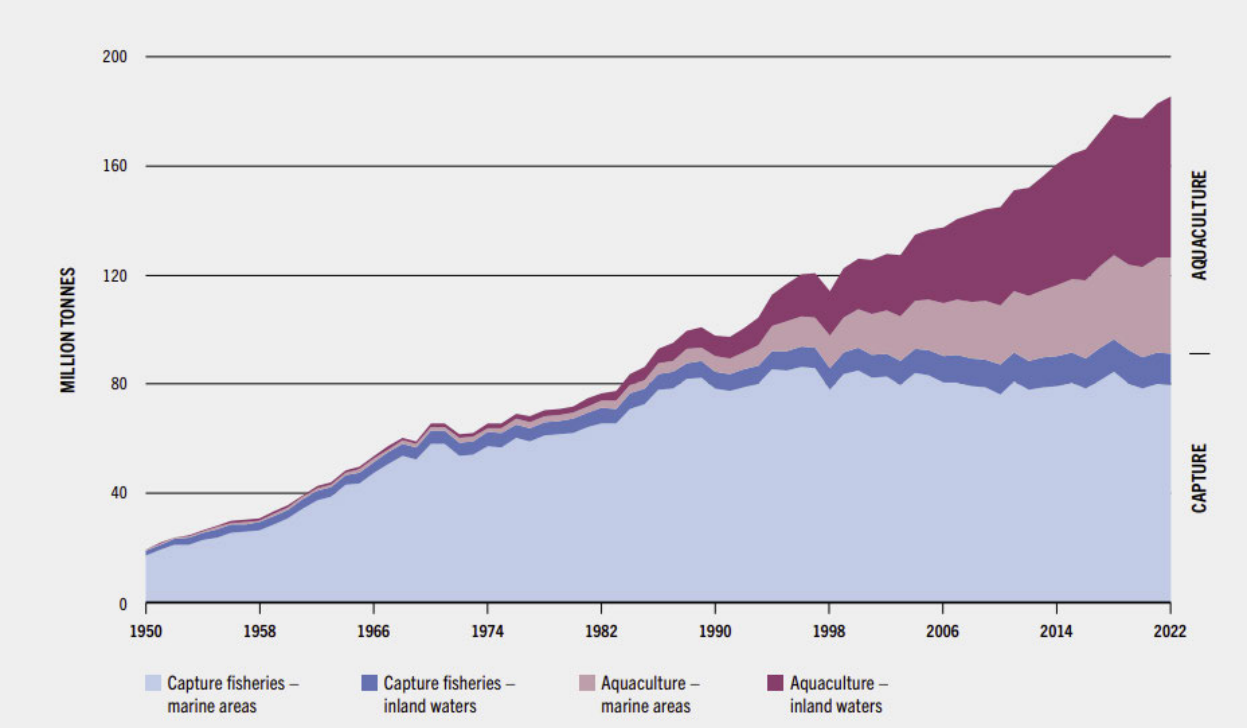


Figure 2: World capture fisheries and aquaculture production from 1950 to 2018. Figure taken from FAO, 2024.

The rapid expansion of aquaculture is closely linked to the planet's increasing population, which has resulted in a greater requirement for food production; particularly sources of protein. The Food and Agriculture organisation (FAO) reported that global fish consumption increased at an average annual rate of 3.1 percent from 1961 to 2017, a rate almost twice that of annual world population growth (FAO, 2020). This highlights the importance of aquaculture in meeting the vast demands for seafood products that come with a rapidly growing population. Fish and other seafood products are a key global protein source, being widely recommended as key sources of micronutrients such as essential fatty acids, which are more limited in terrestrially derived protein sources (Henchion et al, 2017). This nutritional profile is especially important to populations in least developed countries where livestock faming is scarce, making aquaculture-based produce a primary source of protein. For example, 2017 reports show that fish accounted for 50% of the daily protein in Bangladesh, Cambodia, the Gambia, Ghana, Indonesia, Sierra Leone, Sri Lanka and some small island developing states (FAO, 2020; Tidwell and Allan, 2001).

Global population increase is tied to additional pressures such as increased urbanisation, an aging population, and a general increase in global wealth, all of which are predicted to increase protein demands even further (Henchion et al, 2017; FAO, 2020). These pressures drive a demand for diversification of protein sources, which must be produced in a greater quantity as life expectancies increase. Also, in light of climate change, there is substantial pressure to meet these food demands in a way which is environmentally sustainable and can function effectively in resource limited settings. There is already promising research to suggest that the aquaculture industry has the potential to meet these requirements with reports of practices such as bivalve mollusc aquaculture being highly favourable in terms of greenhouse gas emissions (Ray et al, 2019). Therefore, in order to meet food production demands, the development of aquaculture must meet with environmentally as well as socially and economically based sustainability goals (Jennings et al, 2016; Oglend, 2020; Smárason et al 2019).

1.2 The role of bivalve production in aquaculture:

Valued at $323 million and 35% of the total industry value, shellfish production is a significant proportion of aquaculture (Yeh et al, 2020). It is also a particularly diverse sector, accounting

for the cultivation of 104 molluscan and 62 crustacean species (Houston et al, 2020). In keeping with the general trend of aquaculture expansion, shellfish production has been increasing at a rate of approximately 7.8% per year mainly occurring in Asia (Ferreira et al, 2007). Within this varied sector of aquaculture, molluscs are a particularly important phylum, being the second largest category of farmed seafood in terms of both quantity and value (Botta et al, 2020). Arguably the most important grouping of bivalve molluscs to aquaculture are the oysters (family name *Ostreidae*), as they are the most abundantly harvested and cultivated (Trabal Fernández et al, 2014). Oysters are relatively simple to cultivate, and are adaptable to a wide range of latitudes, salinities and temperatures, with some of the most important aquaculture species including the Pacific oyster (*Crassostrea gigas*, now also known as *Magallana gigas*), the American oyster, (*Crassostrea virginica*) and the Sydney rock oyster (*Saccostrea glomerata*). In 2020, cupped oysters of the *Crassostrea* genera were the most produced of the oyster species (FAO, 2020).

In contrast to similar bivalve markets such as mussel production, which has been in decline over the past two decades, the global production of oysters has increased dramatically since the 1950's (FAO, 2022); (Figure 3). An increased demand for oyster production can be expected to continue even further with expectations for mollusc production to reach approximately 22,500 tons by 2030 - an approximate 9,500 ton increase from 2009 data (Kobayashi et al, 2015). This trend, which can be accredited to advances in aquaculture such as improved larval culture techniques and the emergence of triploid oysters have been able to meet the increasing market demands (Botta et al, 2020).

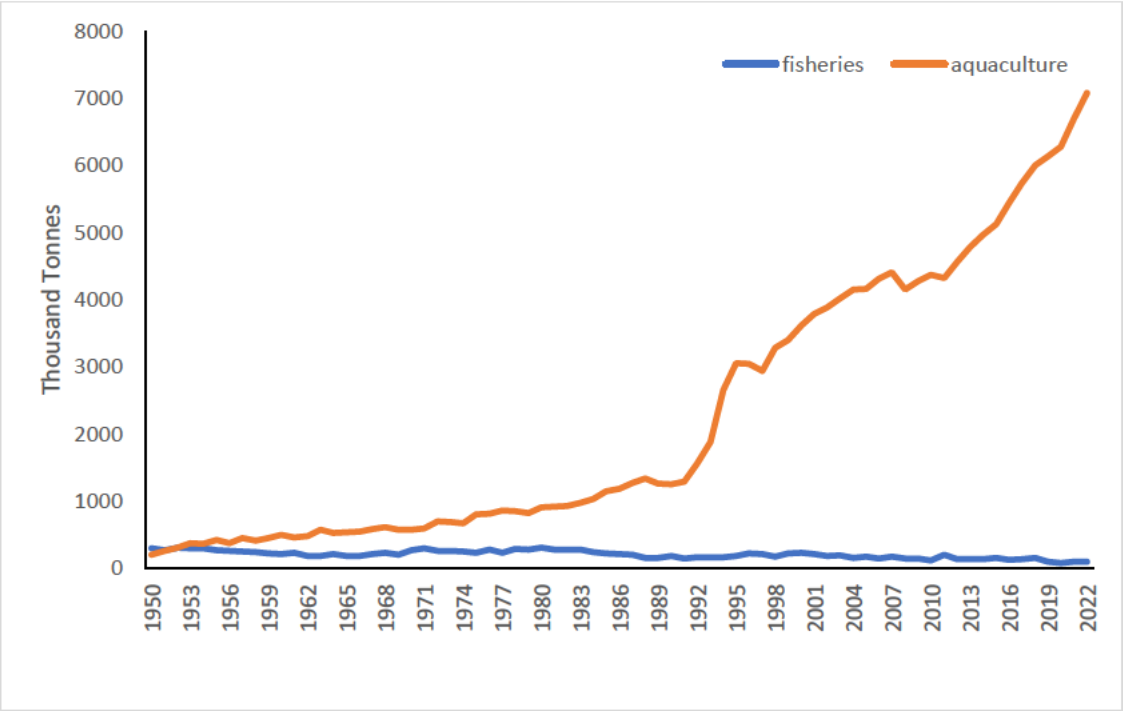


Figure 3: Showing global oyster production since 1950. Figure taken from FAO 2022 report.

C. gigas is one of the most important marine aquaculture species with high production rates of 643.5 thousand tonnes in 2018 (FAO, 2020). The general biology of *C. gigas* has been relatively well studied, which allows for a good understanding of its survival parameters and thus knowledge as to why it is adaptable to such a wide range of environments. Although native to North West Pacific and Sea of Japan (Herbert et al, 2016), the species is now farmed in a wide range of locations after being introduced to the Mediterranean Sea in response to the collapse of native oyster populations in the 1960's (Martínez-García et al, 2021). The cultivation of the species has then expanded further to include locations such as France, the UK, Spain, New Zealand, Australia, USA, and Germany (Galtsoff, 1964; Ezgeta Balić et al, 2020). This broad adaptability is possibly due to important biological traits such as being able to colonise intertidal as well as subtidal zones, a broad temperature survival range (-5˚C to 35˚C) and very few natural predators (Nielsen et al, 2017).

The biphasic lifecycle of *C. gigas* is also well characterised, where development is known to be heavily influenced by temperature and feed rate (Lucas et al, 2019; Rico-Villa et al, 2009; Vogeler et al, 2016; Vogeler et al, 2020); (Figure 4). Each *C. gigas* spawning releases between several thousand through to tens of millions of eggs, or magnitudes more sperm, directly into the water column. Fertilisation then occurs in open water and development of the embryo

progresses through a planktonic phase of the life cycle, (which lasts between 2- 4 weeks) prior to settlement as a sessile animal (Figure 4, stage 2). Roughly 12 hours post fertilization the first free swimming larvae known as a trochophore is seen (Figure 4, stage 6). This then progresses into a D-shaped larva at roughly 24 hours which feeds on particulate organic matter (POM) found within the water using a ciliated lobe referred to as a velum (Figure 4, stage 7). This phase can last between 1 and 4 weeks and as the larvae increase in size from 70 µm in diameter to roughly 300 µm, their diet will progress to diatomaceous algae and then micro algae. The final larval stage (pediveliger) includes the development of a foot (Figure 4, stage 9), which is later lost during the metamorphosis into a spat (Figure 4, sessile stage). This metamorphosis, triggered by specific environmental queues, also sees the development of gills, a gill-based feeding system and byssal threads for attachment to the substrate (Vogeler et al, 2020; Plough, 2018). The spat will then enter a period of growth, during which sexual maturity is reached and the life cycle can begin again. Adult oysters tend to be harvested at 1 – 3 years old but if allowed, can live considerably longer. Importantly, the nature of this lifecycle allows for the controlled management of larval rearing environments in order to select for conditions which optimise oyster production.

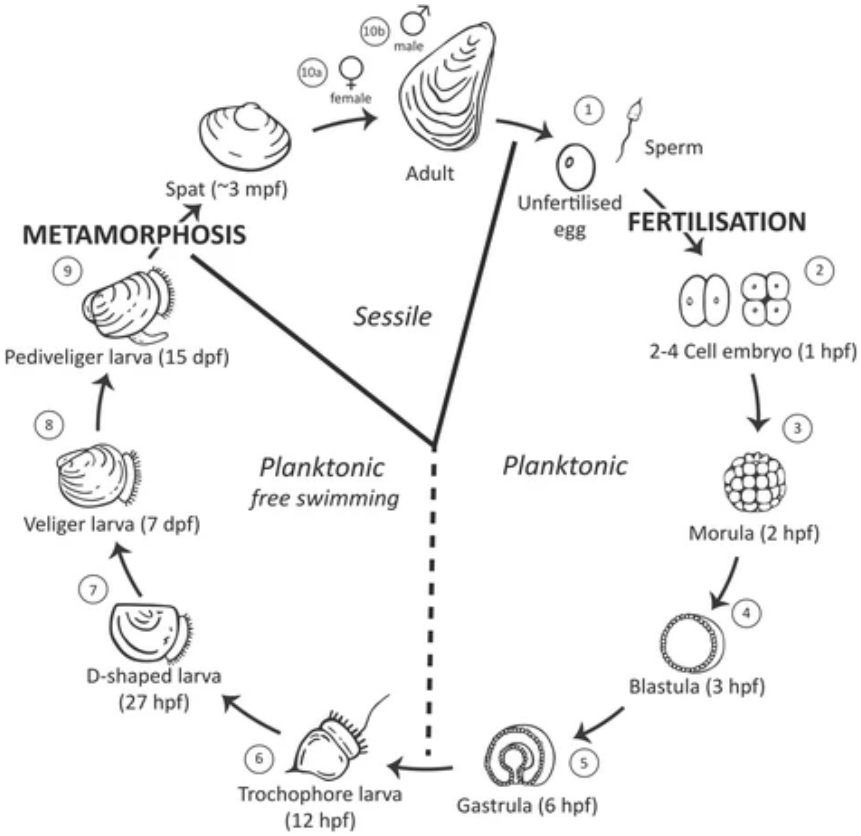


Figure 4: The biphasic lifecycle of *Crassostrea gigas*, showing the progression of the embryo through to the free-swimming larval stages and progression to the spat and final adult stage. Figure taken from Vogeler et al, 2016.

An increased understanding of this lifecycle has allowed for the development of more successful spawning, fertilisation and larval rearing protocols. This in turn has allowed for more efficient cultivation of oysters within hatcheries and resulted commercial rearing being a key method by which *C. gigas* and *O. edulis* (European flat oyster) are produced. Indeed, the majority of cultured oysters produced in China is reliant upon seed stock rather than through the exploitation of wild stocks (Li et al, 2011; Nascimento-Schulze et al, 2021). The establishment of hatcheries has allowed for the cultivation of oysters around the globe, since unlike wild production strategies, seed stock benefit from the closely controlled environment and are not vulnerable to habitat destruction (Nascimento-Schulze et al, 2021).

The lifecycle of the European flat oyster, *Ostrea edulis* (*O. edulis*), is also well described, with key developmental differences highlighted in comparison to *C. gigas* (Colsoul et al, 2021; Helmer et al, 2019; Stechele et al, 2022). Most importantly, *O. edulis* reproduction involves fertilisation within the mantle cavity of the female oyster (Helmer et al, 2019). Larvae then brood within the mantle cavity before being released between seven to ten days post

fertilisation as 'D shells' (Helmer et al, 2019). Unlike *C. gigas* (primarily produced as a global protein source), *O. edulis* is also of particular relevance as the native UK oyster species. It is produced within hatcheries for use within ecological restoration projects, utilising their function of 'ecosystem engineers'. The need for the production of *O. edulis* has been driven by drastic declines in wild populations over the past 40 years which have arisen as a result of habitat destruction and overexploitation (Gundappa et al, 2022). This thesis therefore focuses on the improvement of *O. edulis* and *C. gigas* production, allowing for both food security and ecological applications.

2.0 Main challenges to the aquaculture industry:

2.1 Broad scale challenges:

Whilst aquaculture presents a promising solution to meeting the demands of a rapidly growing population, there are a number of key issues, that challenge the sustainability and continued expansion of the industry. The contributing effects of climate change for example, are expected to have a large impact on the aquaculture industry. These include rising global temperatures and sea levels, more frequent extreme climatic events and water stresses such as a decrease in the availability of fresh water (De Silva and Soto, 2009). The effects of rising temperatures are directly linked to aquaculture species given their poikilothermic nature, meaning that their metabolism and therefore growth rates are likely to be altered. Additional effects might see the relocation of farms in brackish water environments as salinity levels in these areas increase, as well as a focus towards the development of high salinity tolerant strains (De Silva and Soto, 2009). The effects of climate change may also be more widespread and less direct throughout the industry, causing changes to patterns in market supply and demand (Schrobback et al, 2018; Bushek and Ford, 2016).

Additional issues are caused by processes of urbanisation in coastal areas, or “coastalisation”, the increase in human activities and development in coastal areas or cities. Whilst the direct effects of these processes on aquaculture are difficult to record and quantify, they are responsible for habitat destruction, water pollution and pressure on natural resources (Capone and Di Giulio, 2001). Many of these issues are experienced by, (although not exclusive to), the Chinese aquaculture industry and come at a great economic cost with pollution events in 2008 resulting in an industry a loss of 23.6 million Yuan (Li et al, 2011). Some of the main contributing factors to these pollution events include industrialisation, pesticide run-off from agricultural land and conflicts concerning land distribution between aquaculture and other industries such as tourism (Adewolu, 2009; Cole et al, 2009; Li et al, 2011). Through the consequent increased competition for space and natural resources, these pressures place an extra stress on the development of both coastal and inland aquaculture areas.

The presence of environmental contaminants such as pesticides and anti-fouling agents like tributyltin can lead to failed stock declines (Gagnaire et al, 2007; Jones et al, 2006). Some metal ions, if found in excess, can also cause slowed growth and abnormal development of larvae. These are most often introduced via materials used for hatchery fittings, cleaning procedures or seawater storage facilities (Jones et al, 2006). Many of these broader issues which threaten the sustainability of aquaculture are interlinked, for example climate change is likely to cause changes in hydrological regimes in inland waters, which may increase eutrophication events and therefore pollution stress (De Silva and Soto, 2009). Likewise, other challenges such as negligent government regulations can result in the misuse of antibiotics and lax regulation over production methods (Li et al, 2011; Botta et al, 2020).

As is the case with terrestrial farming sectors, disease is the most notable challenge to aquaculture (Bondad-Reantaso et al, 2005; Stentiford et al, 2012). The large-scale production of many key aquaculture species is hindered by disease outbreaks. In the crustacean sector the viral pathogen which causes White Spot Disease in Litopenaeus vannamei (Pacific white leg shrimp), is the greatest challenge to their production, causing global losses of 8 billion since 1990 (Stentiford et al., 2012). In the production of salmon, *Lepeophtheirus salmonis* (salmon louse) is responsible for huge stock declines. The high-density stocking of salmon associated with their aquaculture provides an environment within which the parasite can

proliferate very efficiently. This results in a range of physiological symptoms including skin erosion, osmoregulatory failure and chronic stress, alongside posing a threat to wild stocks via spillback effects (Overton et al, 2019; Torrissen et al, 2013). The most economically significant effect of sea lice occurs through reduced growth rate and poor feed conversion in the fish, which results in a loss of 200g per fish every year until harvest (Mustafa et al, 2001). These disease-based consequences of high-density stocking approaches have arisen alongside the need to commercialise in order to meet food security demands (Bondad-Reantaso et al, 2005).

Disease also poses the most significant threat to shellfish aquaculture, where tools for the prevention of outbreaks are limited (Paillard et al, 2022). Being extrinsically linked to their environment, shellfish are subjected to a wide range of bacterial, viral and parasitic organisms, many of which have the potential to cause disease. Notable diseases amongst oyster species include (but are not limited to) oyster herpes virus (OsHV-1), Vibriosis, Dermo disease and Oyster Velar Virus Disease (Figure 5).

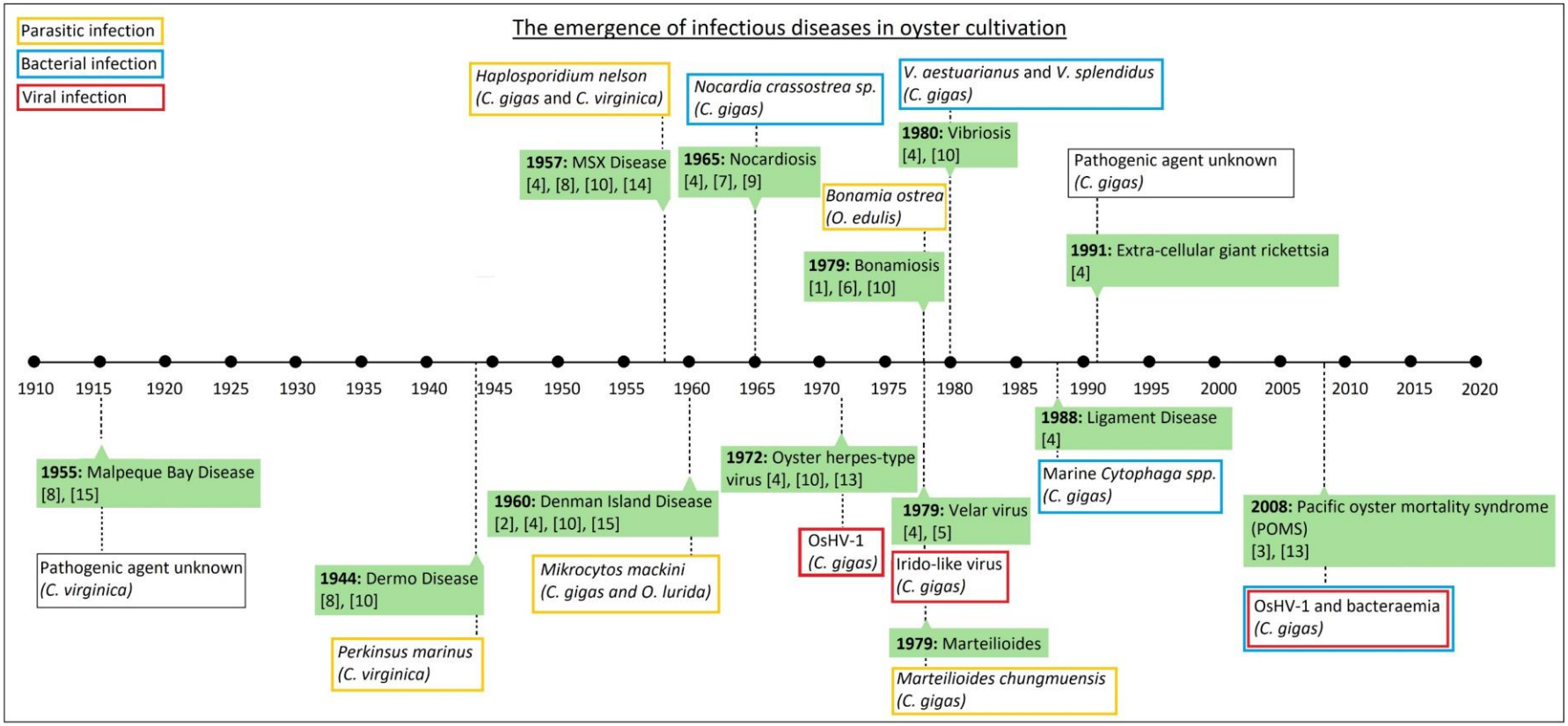


Figure 5: Timeline showing the emergence of some of the most influential infectious diseases within oyster cultivation. The common name of each disease is shown (green box) with its aetiological agent (if known) above the host species in brackets. Parasitic, bacterial, and viral pathogens are highlighted in yellow, blue and red retrospectively.

[1] Arzul, I., Miossec, L., Blanchet, E., Garcia, C., François, C., & Jean-Pierre, J. (2006). Bonamia ostreae and Ostrea edulis : A Stable Host-Parasite System in. Proceedings of the 11th International Symposium on Veterinary Epidemiology and Economics, (1), 6–11.**[2]** Bower, S. M., & Meyer, G. R. (2004). Mikrocytosis (Denmen Island Disease of Oysters) Ch. 5.2.5, (May), 1–8.**[3]** De Lorgeril, J., Petton, B., Lucasson, A., Perez, V., Stenger, P. L., Dégremont, L., … Mitta, G. (2020). Differential basal expression of immune genes confers Crassostrea gigas resistance to Pacific oyster mortality syndrome. BMC Genomics, 21(1), 1–14. https://doi.org/10.1186/s12864-020-6471-x**[4]** Elston, R. A. (1993). Infectious diseases of the Pacific oyster, Crassostrea gigas. Annual Review of Fish Diseases, 3(C), 259–276. https://doi.org/10.1016/0959-8030(93)90038-D**[5]** Elston, R. A., & Wilkinson, M. T. (1985). Pathology, management and diagnosis of oyster velar virus disease (OVVD). Aquaculture, 48(3–4), 189–210. https://doi.org/10.1016/0044-8486(85)90124-3. **[6]** Elston, R. A., Kent, M. L., & Wilkinson, M. T. (1987). Resistance of Ostrea edulis to Bonamia ostreae infection. Aquaculture, 64(3), 237–242. https://doi.org/10.1016/0044-8486(87)90328-0**[7]** Engelsma, M. Y., Roozenburg, I., & Joly, J. P. (2008). First isolation of Nocardia crassostreae from Pacific oyster Crassostrea gigas in Europe. Diseases of Aquatic Organisms, 80(3), 229–234. https://doi.org/10.3354/dao01938**[8]** Ewart, J. W., & Ford, S. E. (1993). History and impact of MSX and dermo diseases on oyster stocks in the Northeast Region. NRAC Fact Sheet, (200), 1–8**.[9]** Friedman, C. S., Beaman, B. L., Chun, J., Goodfellow, M., Gee, A., & Hedrick, R. P. (1998). Nocardia crassostreae sp. nov., the causal agent of nocardiosis in Pacific oysters. International Journal of Systematic Bacteriology, 48(1), 237–246. https://doi.org/10.1099/00207713-48-1-237**[10]** Guo, X., & Ford, S. E. (2016). Infectious diseases of marine mollusks and host responses as revealed by genomic tools. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1689). https://doi.org/10.1098/rstb.2015.0206**[11]** Matsuyama, T., Yasuike, M., Fujiwara, A., Nakamura, Y., Takano, T., Takeuchi, T., … Nakayasu, C. (2017). A Spirochaete is suggested as the causative agent of Akoya oyster disease by metagenomic analysis. PLoS ONE, 12(8), 1–23. https://doi.org/10.1371/journal.pone.0182280**[12]** Matsuyama, T., Matsuura, Y., Inada, M., Takano, T., Nakayasu, C., Sakai, T., … Masaoka, T. (2018). An epidemiological study of Akoya Oyster disease using polymerase chain reaction targeting spirochaetes genes. Fish Pathology, 53(2), 63–70. https://doi.org/10.3147/jsfp.53.63**[13]** Paul-Pont, I., Dhand, N. K., & Whittington, R. J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters Crassostrea gigas. Aquaculture, 412–413, 202–214. https://doi.org/10.1016/j.aquaculture.2013.07.038**[14]** Sunila, I., & Visel, T. (2015). An epizootic of haplosporidium nelsoni in the eastern oysters crassostrea virginica collected from the Connecticut Coastline in 1986 and 1987: An examination of archived slides. Journal of Shellfish Research, 34(2), 297–302. https://doi.org/10.2983/035.034.0211**[15]** Sprague, V. (1971). DISEASES OF OYSTERS, (451), 211–230.

Crucially, oyster production is heavily reliant upon a continuous supply of good quality spat (sometimes referred to as seedstock). Spat can be harvested from natural spatfall or produced in hatcheries, with recent years seeing aquaculture becoming increasingly reliant upon hatchery-produced spat (Botta et al, 2020). The cultivation of *C. gigas* in the UK for example is now entirely reliant upon hatchery-produced spat. Once purchased from an oyster hatchery, spat can then be matured to market size at different locations in cages, bags, or trays placed out in open water. Disease is the most significant limitation to the production of spat within hatcheries, where large scale mortality events cause regular losses of complete batches (Pernet et al, 2012; Pernet et al, 2016). These events are likely caused by a combination of genetic, physiological, environmental and microbial variables, which together have the potential to halt the production of spat that the industry is so reliant upon. Disease events often occur at larval stages of the lifecycle, frequently caused by bacterial pathogens such as *Vibrio tubiashii* and *Vibrio coralliilyticus* (Richards et al, 2014). Should the hatchery process be successful and raise oysters past the larval stages, further diseases such as ligament disease and nocardiosis which act at the juvenile and adult stages of the life cycle may still threaten production (Elston, 1993). New pathogens are also regularly emerging, and whilst diseases of concern have been recognised, they remain without effective treatment (Bui et al, 2019; Stentiford et al, 2012).

In order to work towards resolving these broad scale challenges to aquaculture, additional research must be focused upon the different ways in which disease can arise and possibly be prevented or resolved. One particularly under-researched area of interest is the microbial ecology of oyster species such as *C. gigas* and *O. edulis*. This includes studying the microbial species associated with the oysters themselves, their hatchery environments, and relationships between the two. Current literature does include some studies (Arfken et al, 2021; Trabal Fernandez et al, 2014; King et al, 2019a; King et al, 2012; Ossai et al, 2017; Schulze et al, 2006), but many important questions such as how these microbes are acquired and change over time are left unanswered. It is also important to understand the role of these microbes in the overall health and biology of the species, particularly in the early larval stages of life. This understanding would then likely act as foundation to investigate the ways in which the microbiome is linked to the progression of disease and could be manipulated in favour of disease mitigation strategies.

The investigation of the microbes in commercial oyster species and their hatchery environment is therefore an important area of research which has great potential to reduce spat mortality. This review will therefore discuss the current literature around microbiome studies in oyster aquaculture and identify the most important knowledge gaps to address in further research.

3.0 The microbiome:

3.1 The importance of studying the microbiome:

The term microbiome describes the collection of microbes including archaea, bacteria, fungi, protists and viruses that are associated with an animal or plant host or that occupy a particular environment (Zhu et al, 2023). Since the early 2000's, approaches to studying the microbiome have advanced rapidly alongside an increasing awareness of the importance of microbiome-host interactions. This has seen microbiome studies become increasingly well reported within the scientific literature, covering a wide range of host species of medical, ecological and environmental importance. Although initial large-scale microbiome studies have been more heavily focused upon human health applications (Proctor, 2011; Turnbaugh et al, 2007; Ursell et al, 2012), the ever-decreasing cost of next generation high throughput sequencing (HTS) has allowed for microbiome research to expand much further in its applications to all areas of research, including aquaculture. This is a promising step forward for the industry, allowing the investigation of developmental and rearing processes in key species to be advanced significantly.

The importance of the microbiome to the health and development of other major aquaculture species have been more widely reported in recent years. The role of the gut microbiome in channel catfish, largemouth bass and bluegill have been stated as influential in the development of the gut epithelium as well as providing essential nutrients and stimulating the innate immune system (Larsen et al, 2014). Similar roles of the microbiome have been reported in invertebrate studies, with beneficial bacteria in Pacific Whiteleg shrimp promoting nutrient intake as well as conferring resistance against pathogens (Md Zoqratt et al, 2018). Alongside host-based studies, the role of environmental microbiomes within

aquaculture has been briefly considered, with evidence demonstrating the bacterial species present in tank water to be a more insightful indicator of water quality compared to other common measurements such as temperature, pH, salinity or oxygen concentration (Md Zoqratt et al, 2018).

The scope of microbiome studies within marine invertebrates are somewhat varied, covering host Phyla from *Cnidaria* (Fraune and Bosch, 2007; Tout et al, 2014) through to *Porifera* (Fan et al, 2012). These studies have been successful in reporting commensal bacteria which are distinct to their environment, and highly conserved over time in both wild and laboratory kept settings (Pierce and Evan, 2019). Such studies suggest that the host does impart some control over microbiome composition. It is also widely recognised that microbiomes within invertebrates can be tissue specific, with many studies suggesting an important role of the gut microbiome in the overall health of the host species (Gao et al, 2020; Mattila et al, 2014; Rasheeda et al, 2017).

The role of the microbiome in different oyster species has been increasingly well researched in more recent years, (Cho et al, 2024; English et al, 2023; Scanes et al, 2023; Unzueta-Martínez, 2022; Zhong et al, 2024). The microbiome of *C. gigas* for example may play beneficial roles such as aiding development of the gastro-intestinal (GI) tract and inhibiting colonisation by pathogenic microbes (Arfken et al, 2021; Prado et al, 2010; Schulze et al, 2006). This indicates that *C. gigas* microbiome research could have impactful applications and be of benefit to cultivation e.g., improved quality of seed stock and development of disease mitigation strategies. As such, it is important to expand research of the *C. gigas* (and other oyster species) microbiome interactions to fully understand the possible benefits that this could lead to for the aquaculture industry.

3.2 Current methods:

As is the case with many microbiome studies, investigation of the oyster microbiome was originally dependent on microbial culture methodologies. These were conducted throughout the 1960's and largely investigated the microbiome in relation to larval and adult disease. Murchelano & Bishop, 1969 conducted one of the first studies aimed at identifying the bacterial species present in healthy juvenile *C. gigas* individuals. Here, the morphological and physiological characterisation of colony forming units (CFUs) grown from bacterial isolates

were used to report the presence of genera such as *Archromobacter*, *Bacillus*, *Cytophaga*, *Flavobacterium*, *Pseudomonas* and *Vibrio*. Prior to this, similar methodologies had been used to investigate the microbiome of healthy adult oysters, with the aim of such work being a necessary prerequisite to disease-based challenge experiments (Colwell and Liston, 1959). The nature of these studies makes them limited in a variety of different ways, as discussed by Murchelano & Bishop, 1969. For example, variations in the isolation techniques and taxonomic schemes used are highly likely to introduce bias through favourably selecting for certain bacterial species and ruling out any present at a particularly low abundance (Murchelano and Bishop, 1969). These early studies were successful in identifying the bacterial species present in oysters and their larvae, but the functional roles of these bacteria remained undiscussed. The development of techniques which allowed for the creation of bacteria-free (axenic) *C. gigas* larvae (Douillet and Langdon, 1993) did however allow for the research where different bacterial strains were proven to be detrimental or beneficial to host health.

In contrast to culture dependent studies, the development of HTS (high-throughput sequencing), has allowed microbiome studies progress rapidly and cost efficiently via the amplification and sequencing of specific markers (metabarcoding) (Infante-Villamil et al, 2020; Ogier et al, 2019). The vast majority of microbiome studies now use a 16S rRNA based technique, with this ribosomal coding gene being the section of DNA most commonly sequenced as a "barcode" for prokaryotic identification (Clifford et al, 2012; Mignard and Flandrois, 2006; Woo et al, 2008). Many of these studies follow a similar laboratory-based pipeline, with variations mostly arising from differences in the type of sequencing used and region of the 16S gene to be amplified. For example, past studies show evidence of pyrosequencing and Illumina sequencing based approaches with amplification of hypervariable 16S regions ranging from V1-V6 (Dupont et al, 2020; King et al, 2012; Trabal Fernández et al, 2014). In contrast to 16S-based methods, sequencing of the 18S rRNA gene allows for the identification of eukaryotic species, with the V4 region being the most variable and frequently sequenced (Hadziavdic et al, 2014). This method has highlighted *Labyrinthulomycetes* and *Chlorarachniophyta* as core eukaryotic classes within the microbiome of Pacific oyster spat (Zhong et al, 2024).

Alternative sequencing techniques include the use of Nanopore technologies, which allow for the full-length sequencing of the 16S region. Whilst records of Nanopore technologies being used for 16S work within oyster species are limited, the *O. edulis* genome has been reported using the PromethION (from Oxford Nanopore Technologies); (Gundappa et al, 2022). Long-read sequencing is advantageous in that it allows for increased taxonomic resolution over sequencing hypervariable regions of the 16S rRNA gene, in theory resulting in a more accurate representation of the microbiome. However, in practice, Nanopore technology is often criticised for its high error rate; around 10% in comparison to a 1% of short-read platforms (Nicholls et al, 2019; Rang et al, 2018). This error rate is however been continuously improved upon with the development of new laboratory equipment and basecalling software (Mackenzie and Argyropoulos, 2023). For example, the basecalling software Guppy (V3.6) is 97% accurate - a 1-2% improvement of its previous version (Huang et al, 2021). More recent studies opt for the basecalling software Dorado (v5), giving a 99.75% accuracy (McGrath et al, 2024; Zidane et al, 2024). A long-read sequencing approach was chosen throughout this thesis in order to increase taxonomic resolution in comparison to short-read methods, whilst also remaining more cost effective than whole-genome shotgun sequencing. This ultimately enabled a greater number of samples to be processed and more extensive data analysis. Descriptive microbiome studies routinely use universal 16S primers, designed to amplify a broad range of bacterial taxa. This thesis follows a similar approach, but with modified universal primers designed to reduce amplification bias against *Vibrio* species – improving the detection of this biologically important genus within the oyster microbiome.

Due to the fact that 16S rRNA based approaches are now so commonly used, they have become advantageous in that this marker gene now has an extensive taxonomic database which can allow for identification of bacteria down to the genus and species level for many but not all species. Whilst this is useful for analysing shifts in microbial communities, or for identifying static communities, the inability to definitively identify taxa to species level means that whole genome sequencing (WGS) and transcriptomics are still often necessary (Ogier et al, 2019). High similarities between 16S sequences can also sometimes lead to false matches being made against the databases, resulting in the reporting of a species or taxa to be incorrectly present or poorly discriminated between (Ogier et al, 2019). With fewer 16S based studies having been conducted in aquatic environments than other environments, many

species within these microbial communities currently remain uncharacterised. Gaps within the taxonomic databases for this environment therefore present a challenge to aquatic studies. Further research using this marker gene is crucial in order to expand upon these databases and allow for more effective identification.

These limitations have led to the need for the development of alternative universal bacterial primers, such as those for a specific portion of the housekeeping rpoB gene (Ogier et al, 2019). Studies comparing the use of the rpoB gene as a genetic marker against 16S rRNA have found it to be more effective at assigning organised taxonomic units (OTUs) of a known mock community to a species level. Such findings have led to suggestions that microbiome studies would become more comprehensive when analysis is carried out using both the 16S rRNA gene and another gene as biomarkers (Ogier et al, 2019). In terms of studying the *C. gigas* microbiome specifically, the inability to consistently identify OTUs down to a species level with 16S based approach is a particularly significant limitation. This is because species of the *Vibrio* genus are particularly important, with some species naturally residing within the host whilst others such as *V. splendidus* and *V. aestuarianus* are known to have a detrimental effect to oyster health (Bean et al, 2024). This has led to the development of alternative techniques such as the use of heat shock protein (hsp60) as a phylogenetic marker in order to identify *Vibrio* down to the species level (King et al, 2019b).

3.3 Core and non-pathogenic species of the microbiome:

Within microbiome research generally, the core microbiome can be defined in a number of different ways with a focus on: common, temporal, ecological, functional or host-adapted taxa (Risely et al, 2020). Previous oyster larval studies have followed core microbiome definitions based on common taxa – possibly because other parameters such as functionality remain under researched within oyster species. There is however a large variability in the stringency with which the parameters for core oyster larval microbiome are set, with definitions ranging from OTUs which are shared among all larvae (Arfken et al, 2021) or more than 50% of spat (Cho et al, 2024). Both of these studies did however both report core taxa belonging to the Rhodobacteraceae and Flavobacteriaceae bacterial families. Risely et al, 2020 notes that studies of core microbial taxa enable the identification of microbes which drive ecological and evolutionary processes within a host population. In oyster populations,

this may be seen through core taxa conferring resistance to stressors such as heat shock events or opportunistic pathogens. Although not explored within the current literature, this may provide ecological and evolutionary benefits through improving resilience and adaptability. Taxa belonging to the core larval microbiome are the most likely to be functional, with roles beneficial to development, disease resistance and maintenance of overall health (Arfken et al, 2021; Pimmentel et al, 2021; Risley, 2020). It could therefore be suggested (although not yet proven), that the establishment of a core microbiome is necessary in order to maintain larval health.

In the interest of maintaining a stable larval microbiome, hatcheries routinely practice techniques such as water filtration and UV treatment which reduce the bacterial load and richness to which larvae are exposed to (Wallace et al, 2008). Such practices are necessary in order to reduce the risk of bacterial infection. They may also be beneficial in reducing the energetic costs required by larvae to maintain a stable microbiome (Gillingham et al, 2024). A completely sterile environment may however hinder the establishment of taxa which are beneficial to larval health. Regardless, the maintenance of a completely sterile hatchery environment is an unrealistic goal for commercial or research-focused hatcheries.

As filter feeding animals, with partially open circulatory systems, the surrounding environment is expected to influence the core, non-pathogenic members of the oyster microbiome. In *C. virginica* for example, the gut microbiome has been reported to share similarities to that of its surrounding seawater and marine aggregate (particles of organic matter); (Pierce and Ward, 2019). Bacterial orders of Alteromonadales, Brachyspirales, and Synechococcales were found within each of these samples types (Pierce and Ward, 2019). The presence of OTUs belonging to Flavobacteriales and Sphingobacteriales in seawater or marine aggregate samples did not however guarantee their transfer to *C. virginica* (Pierce and Ward, 2019). Pierce and Ward, 2019 also defined core microbiomes of these sample types, defined as OTUs shared across 95% of samples analysed. Importantly, *C. virginica* shared 117 core OTUs with the gut microbiome of *Mytilus edulis*, compared to just 70 shared with marine aggregate – highlighting consistent microbial differences between sample types.

Stevick et al, 2021 also compared microbial profiles between the gut of *C. virginica* and the surrounding seawater. Similarly, many common taxa were found between the two sample types (characterised into 135 different bacterial orders), however an additional 161 were

found exclusively in the gut (Stevick et al, 2021). In studies of adult *C. gigas*, the oyster microbiome has also been found to be distinct to that of the surrounding seawater, with less than 21% of bacterial genera to be shared between the two (Liu et al, 2024). These findings suggest that the host, or the microhabitat within the host imparts a level of selection, making the gut microbiome distinct from the surrounding water. This selective process is hypothesised to be implemented via feeding selection, niche colonisation or evasion of immunity (Dupont et al, 2020). When comparing the studies discussed above (Liu et al, 2024; Pierce and Ward, 2019 and Stevick et al, 2021), key methodological differences should be considered. Pierce and Ward 2019 for example incorporated a sample collected process which spanned one year, allowing for the observation of temporal variation within the *C. virginica* microbiome. It is therefore likely that differences in key findings between studies may be influenced by the season within which samples were collected, and similarly, species, location or life stage.

Such baseline comparisons between the oyster microbiome and the external environment will be crucial in elucidating how the oyster microbiome is acquired and maintained; and could have a wide range of implications for oyster health and disease control. The current literature states a broad range of possible sources of the microbiome ranging from horizontal transmission between animals (via the water column), acquiring bacteria vertically from the parents, or oysters retaining and amplifying bacterial species directly from the water column (Dupont et al, 2020).

The majority of existing microbiome studies are focused on the gut of *Crassostrea* species, a sample type which includes the stomach and sometimes also the hindgut (Atker et al, 2024; Dubé, et al, 2019; King et al, 2012; Kong et al, 2022; Pierce and Evan, 2019; Stevick et al, 2021). Other studies also include the whole digestive gland (or hepatopancreas; Schulze et al, 2006) and haemolymph, (Dubé et al, 2019; Dupont et al, 2020; Fu et al, 2024; Schulze et al, 2006). Each study suggests the microbiome of *Crassostrea* to be a highly diverse and dynamic system, with reports of bacteria over a wide range of phyla. The *Proteobacter* and *Tenericutes* phyla are for example highly abundant in the gut microbiome of *Crassostrea virginica* (Pierce and Evan, 2019). This identification was also able to be completed down to a more specific taxonomic level, reporting orders of Mycoplasmatales, Verrucomicrobiales, Fusobacteriales and Brachyspirales as some of the most relatively abundant.

Composition of the gut and adductor muscle microbiomes share common factors (King et al, 2019). For example, King et al, 2019c reports the adductor muscles of non-disease effected *C. gigas* individuals to host species belonging to the Spirochaetes phylum, Brachyspiraceae family and Mycoplasma genus (in order of relative abundance). The microbiome of the haemolymph is also known to share common phyla with the gut microbiota such as Proteobacter and Actinobacter (Dupont et al, 2020). Importantly, Dupont et al 2020, also reports the presence of protists such as Alveolata within the *C. gigas* haemolymph as well as viruses belonging to the Siphoviridae and Myoviridae families. This reporting of eukaryotes and viruses within the microbiome is less well covered within the literature, with current studies mainly focusing on the bacterial species present.

Although relatively little is known about the functionality of specific or individual species within the microbiome, a close relationship between microbe function and the environment is evident (Stevik et al, 2021). Here, an upregulation in genes associated with stress response and phosphorous metabolism in the gut bacteria of *C. virginica* was seen in areas of higher nutrient density and anoxia. Other possible functions within the microbiome include the role of *Mycoplasmatales* which are known in other aquatic species to play a key role in the establishment of biofilm. Species of the genus *Phaeobacter* (found in the gut of *C. virginica)*, may have probiotic effects in protecting the host against *Vibrio* and *Roseolovirus* pathogens (Dupont et al, 2020; Pierce and Evan, 2019). Since the relationship between different microbes, the host and the environment are highly complex, this can make the functionality of microbes difficult to investigate. It is however an important area of research to pursue, as understanding microbe functionality would better inform knowledge of oyster health and therefore has the potential to greatly improve the hatchery success.

3.4 Pathogenic species of the microbiome:

As previously discussed, large-scale mortality events within seed stock are one of the biggest limiting factors to commercial oyster production (King et al, 2019d; Gray et al, 2022; You and Hedgecock, 2019). Whilst the specific causes of these mortality events are most likely multifactorial and often remain unknown (Grey et al, 2022), various bacterial, viral and parasitic pathogens have been identified as the primary causes of some of these outbreaks (Dubert et al, 2017; Elston, 1993; Farley et al, 1972; Pernet et al, 2016; Richards et al, 2015

and Stentiford et al, 2012). More specifically, pathogenic microorganisms have the potential to implement a state of dysbiosis within the microbiome. Described as 'an imbalance or shift in the naturally present microbiome compared to that of a healthy individual' (Infante-Villamil et al, 2021) a state of dysbiosis frequently correlates with a state of disease. Vignier et al, 2021 characterises one mechanism of dysbiosis as an increase in species richness (alpha diversity). This is a commonly used metric within of microbiome analysis which calculates the species richness (number of unique bacterial taxa) within a sample. An increased species richness was associated with impaired *C. gigas* larval health (Vignier et al, 2021). Conversely, Dai, et al, 2024 concludes increased species richness levels to be beneficial to larval health, suggesting this this metric could be used as a biomarker of host fitness. To further investigate these conflicts within the literature, future studies may choose to investigate the effects of reduced species richness on susceptibility to key oyster pathogens.

Dysbiosis-driven disease events often cause huge declines in stock production, sometimes even resulting in the complete collapse of a particular species. This was seen with the collapse of *Crassostrea angulata* stocks in France, which after suffering from a series of infectious disease outbreaks resulted in the species being replaced with *C. gigas* (King et al, 2019d; Roch, 1999). Importantly, mass mortality events at the larval stage of the hatchery process are a regular occurrence (Génard et al, 2011). For example, larval crash events of *C. virginica* have are reported alongside microbial dysbiosis characterised by a dominance of microeukaryotes (Cram et al, 2024). This places a particular importance on investigating the microbiome of oyster larvae, as future disease mitigation strategies would be best targeted at this life stage.

The severity of diseases effecting *C. gigas* has dramatically increased over the past 20 years (Lemire et al, 2015). For example, in 2008, large scale mortality events were reported in France, the United Kingdom, Jersey, Ireland, Spain, the Netherlands and the United States (Paul-Pont et al, 2013). Although the exact aetiology is not completely understood, these mortality events were reported to be caused by interactions between a particular variant of the Ostreid herpesvirus (OsHV-1 µvar) with other pathogens – including bacteria of the *Vibrio* genus (Clerissi et al, 2020; King et al, 2019d; Paul-Pont et al, 2013; Petton et al, 2021). From 2010 onwards, this combination of interactions with OsHV-1 µvar became known as Pacific oyster mortality syndrome (POMS); (Paul-Pont et al, 2013). Today, this disease is a prominent problem within the cultivation of *C. gigas*, occurring worldwide and causing high mortality

rates amongst juvenile oysters (French wild and cultivated stocks are depleted by at least 35% each year); (Petton et al, 2021). OsHv-1 driven mortality events in Australia and Tasmania have been correlated with increasing temperatures (Green et al, 2019; De Decker and Saulnier, 2011; Wendling et al, 2014). This is likely due to a combination of both increased pathogen virulence and occurrence, as well as compromised immunity from the host (Green et al, 2019).

The current literature of POMS commonly reports it to be a multifactorial disease with a complex aetiology, being affected by interactions between a range of different pathogens, host genetics and environmental conditions – most notably seawater temperature (Clerissi et al, 2020; Delmotte et al, 2020; Petton et al, 2015; Petton et al, 2021). The progression of POMS characteristically begins with an OsHV-1 µvar infection which causes immunosuppression within the oyster, allowing colonisation of opportunistic bacteria in various tissues which then becomes fatal (de Lorgeril et al, 2018); (Figure 6). The most commonly studied of these opportunistic bacteria are *Vibrio* species belonging to the *Splendidus* clade, however those belonging to the *Acrobacter*, *Psychromonas*, *Marinomonas*, *Marinobacterium*, *Vibrio*, and *Pseudoalteromonas* genus are also present (de Lorgeril et al, 2018; Lucasson et al, 2020; Richard et al, 2021). Recent findings report *Vibrio* species to be key to the development of systemic POMS infections through causing haemocyte lysis within the oyster, and thereby dampening the cellular defences of the host (Petton et al, 2021). Alongside these studies, current knowledge around the genetic diversity of OsHV-1 is increasing (Morga et al, 2021), which are promising steps to understanding the true dynamics of POMS pathology.

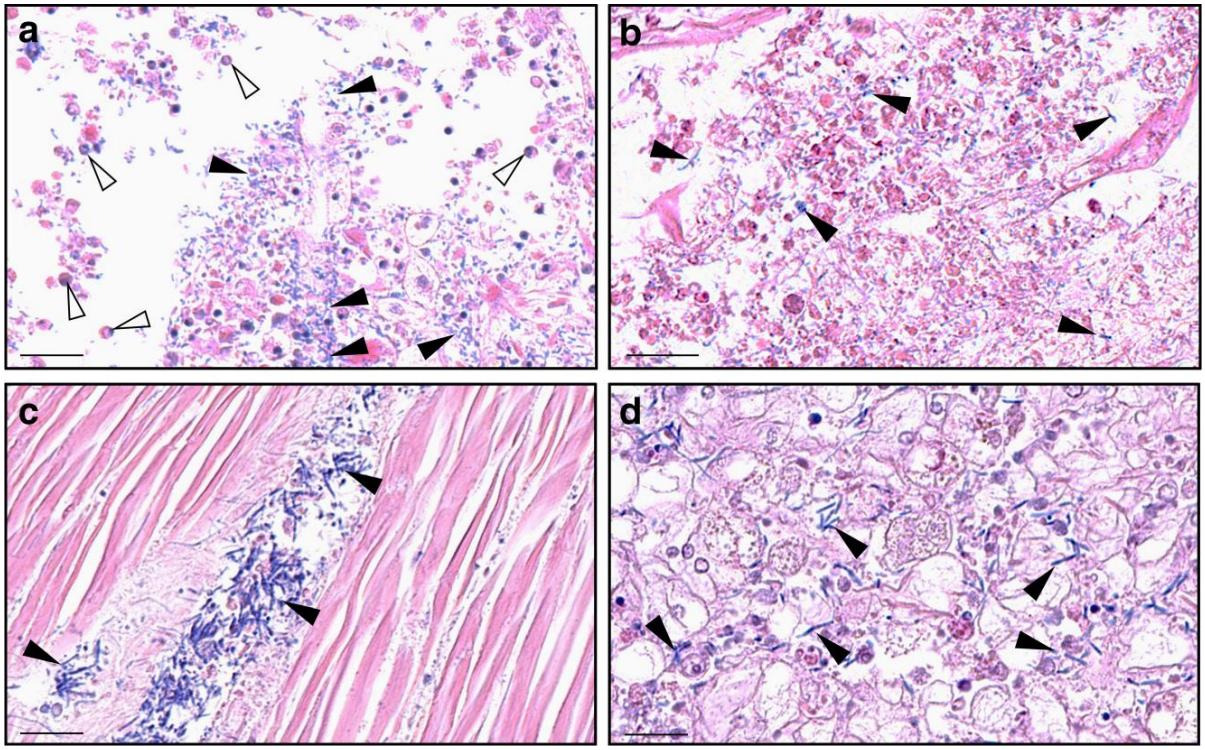


Figure 6: Histological analysis of a. gill tissue in early infection b. gill tissue in late infection c. adductor muscle in late infection and d. interstitial tissues in late infection in juvenile *Crassostrea gigas* individuals challenged with *Ostreid herpesvirus*. The association of the disease with bacteraemia can be seen with the presence of purple stained cells indicated with arrows, (scale bars corresponds to 20 µM). Filled arrowheads indicate bacteria accumulated in the gills. Open arrowheads indicate haemocyte-like cells found in gill tissues and outside tissues in the vicinity of bacteria. Figure taken from de Lorgeril et al, 2018.

Outside of the context of POMS, pathogenic *Vibrio* species are also responsible for the occurrence of vibriosis – one of the most significant diseases to effect wild and hatchery seedstock populations. *Vibrio* bacteria naturally reside within the aquatic environment and whilst vibriosis has been reported on since 1980, the disease remains untreatable. Although the exact role and function are yet to be described, *Vibrio* species have been recorded in the microbiome of healthy oysters (Asmani et al, 2016). Specific *Vibrio* species, including but not limited to, *V. aestuarianus*, *V. coralliilyticus*, *V. neptunis* and *V. tubiashii* can however be responsible for significant seedstock crashes (Dubert et al, 2017; Richards et al, 2015). Between 2006 and 2007, *V. tubiashii* alone was responsible for larval losses of 59% in various bivalve species along the US Pacific coast (Richards et al, 2015).

The complex pathogenicity of vibriosis makes the disease both difficult to treat and map in terms of future outbreaks. Sainz-Hernandez and Maeda-Martinez, 2005 were able to identify sources of *Vibrio* contamination in a scallop hatchery as broodstock (more so from female

gonads), mass-produced microalgae and contaminated tap water. Such studies are uncommon within the current literature but crucial in order to better inform disease mitigation strategies and limit expenses for hatcheries. Future similar research would benefit from using culture independent techniques which would allow for species identification of the *Vibrio* species present.

Oyster populations are likely to show natural varying levels of disease susceptibility, but through selective breeding programmes, increased disease resistance against OsHV-1 µvar infection is possible (Dégremont, 2011; Dégremont et al, 2015). This shows a strong genetic link to disease susceptibility, but the possible role of the microbiome should also be considered (King et al, 2019d; Petton et al, 2015). Resistance against OsHV-1 in *C. gigas* is associated with specific OTUs belonging to the *Winogradskyella* genus and Bradyrhizobiaceae family (King et al, 2019d). The role of *Vibrio* bacteria is crucial to the multi-pathogenic nature of an OsHV-1 infection, with the virus itself not being sufficient to cause mortality in *C. gigas juveniles* (Petton et al, 2015). These studies highlight an important relationship between the microbiome and disease outcome, where future research has great potential applications for aquaculture through reducing the severity of disease mortality events.

4.0 Influencing factors of the microbiome:

4.1: Source of the microbiome:

As with many species, influencing factors of the oyster microbiome are closely related to its main source (or sources). Current research does not suggest a single or definite source of the oyster microbiome, and conclusions vary on the significance of genetic (section 4.2) and environmental influences (section 4.3). Some of the most commonly discussed possible sources of the microbiome include the surrounding sea water, algal feed (section 4.4), and vertical transmission from the eggs used in spawning.

As sessile filter feeding organisms, oysters are intrinsically linked to their environment and the microbes found within it. It can therefore be hypothesised that the acquisition of the bivalve microbiome is more heavily influenced by their external environment (sea water), than in comparison to other species. In the early developmental stages of *Saccostrea*

glomerata, the larval microbiome is dominated by the Alteromonadales Order – found also to dominate the water microbiome (Unzueta-Martínez et al, 2022). Common bacterial classes of *Alphaproteobacteria Gammaproteobacteria*, and *Flavobacteriia* have also been reported to exist in both seawater and *C. gigas* larvae (Arfken et al, 2021; Laroche et al, 2018). This suggests that the surrounding seawater is a likely source of the larval microbiome – particularly within the earlier developmental stages. As *S. glomerata* larvae progress through development, current research reports a more complex microbiome which dominated by Cellvibrionales, Vibrionales and Flavobacteriales (orders which were not detected within the water microbiome); (Unzueta-Martínez et al, 2022). Similarly, in 15-day old *C. virginica* larvae, the microbiome has been found to be dominated by two species of *Betaproteobacteria* (45%), a bacterial family not found within the seawater (Arfken et al, 2021). This implies that sea water may be a greater source of the larval microbiome in the very early life stages before other taxa become more established and suggests an alternative source to the microbiome other than the surrounding sea water.

One possible alternative source are the eggs from the maternal oyster (Unzueta-Martínez et al, 2022). An ASV (amplicon associated variant) belonging to the order *Alteromonadales,* was found in five different larval stages of *Saccostrea glomerata* (Sydney rock oyster), and its source traced back the gametes of parental oysters, with the egg making a greater contribution than sperm (Unzueta-Martínez et al, 2022). This finding is especially important from an aquaculture perspective (see section 4.4), where hatcheries must select oysters to condition for broodstock purposes. Additional *Alteromonadales* ASVs are shared between seawater and early D-veliger larvae, but not in larvae of later ages (Unzueta-Martínez et al, 2022). This further supports the hypothesis that seawater makes a greater contribution the larval microbiome during the early larval stages, but less so in later stages.

Overall, there is sufficient evidence to prove that environmental and parental sources contribute to the oyster microbiome. When determining microbial sources, studies such as Unzueta-Martínez et al, 2022 and Arfken et al, 2021 which track microbial composition throughout larval development or longer are especially important in determining the source of core vs transient taxa. Further work by Unzueta-Martínez, 2022 into the adult stages of *C. virginica* concluded that gill microbiomes were significantly different depending upon the site from which they were sourced, and remained so throughout a 6-week common garden

experiment. The source of the oyster microbiome is therefore most likely shaped by complex interactions between both genetic and environmental conditions.

4.2 Genetic influencing factors:

It is widely reported that genetic factors impart some control over the oyster microbiome (King et al, 2019; Paillard et al, 2022; Pimental et al, 2021; Scanes et al, 2021a; Unzueta-Martínez et al, 2022; Vignier et al, 2021 and Wegner et al, 2013), with key papers relating hypotheses to metabolic rate (Scanes et al, 2021a) and disease susceptibility (King et al, 2019 and Wendling et al, 2017). This work is especially important if oyster breeding programmes go on to employ genomic selection as seen in more established industries (Houston et al, 2020) such as Atlantic salmon (Tsai et al, 2017) and Pacific Whiteleg shrimp (Luo et al, 2022) to allow for increased growth rate or resistance to key pathogens such as OsHV-1 (Boudry et al, 2021). Investigating the relationship between the host genome and microbiome therefore not only adds to general biological understanding, but may also have wider applications of ensuring successful breeding outcomes.

Microbiome-genotype interactions have also been investigated by testing the hypothesis that host processes such as metabolic rate (MR), drive changes in the microbiome in response to climate change (Scanes et al, 2021a). This study recorded microbial changes in the haemolymph of oysters from 9 different genetic lineages in response to treatments of ambient and elevated temperature and partial carbon dioxide (pCO_2 i.e., dissolved CO_2). While the genetic relatedness between these 9 different lineages was not reported, they were known to display different levels of resilience to climate change stressors. A negative relationship was found between MR and species richness; a significant decrease in *Arcobacteraceae* and *Flavobacteriacae* occurred alongside an increasing MR. Although MR in other species is known to have a significantly heritable genetic influence, this result was not dependent upon genotype (Pettersen et al, 2018). Some genotype dependent findings were, however, reported such as decreased bacterial richness when levels of extracellular pH and CO_2 (metabolic by products) increased. Although no definite relationship was reported, this research does highlight the importance of considering microbial change through genetically controlled host processes.

Microbiome-genome studies in oysters have also been carried out within the context of disease resilience. Wendling et al, 2017 investigated genomic control through interactions between the host genotype, pathogen genotype and environment (a GxGxE study). Here, the resistance of *C. gigas* larvae against infection with *Vibrio splendidus* was found to be dependent on host genotype. This provides strong evidence that resistance against bacterial infection has a genetic basis. This is a conclusion supported by King et al 2019 which reported how breeding for resistance against diseases such as OsHV-1 effects the microbiome of *C. gigas*. King et al, 2019 defined core microbial taxa of oysters with different susceptibilities to OsHV-1. Different bacterial taxa were found between varying disease susceptibilities, for example OTUs assigned to the *Photobacterium*, *Vibrio*, *Aliivibrio*, *Streptococcus*, and *Roseovarius* genera were associated with low disease resistance. This provides strong evidence that the host genome does impart some control over the bacterial taxa present and therefore selective breeding may impact the core microbiome.

4.3 Environmental influencing factors:

The environment in which an oyster is grown has important control on its microbiome. From a broad perspective, Bass et al, 2019 suggests that that research questions should be considered from a more holistic point of view through the pathobiome approach. This approach considers the full set of host-associated organisms including prokaryotes, eukaryotes and viruses, as well as how they interact with their environment (Figure 7). This process provides a better understanding of the microbiome, it's functionality in the host and the factors which influence it, although it is not always feasible (Bass et al, 2019).

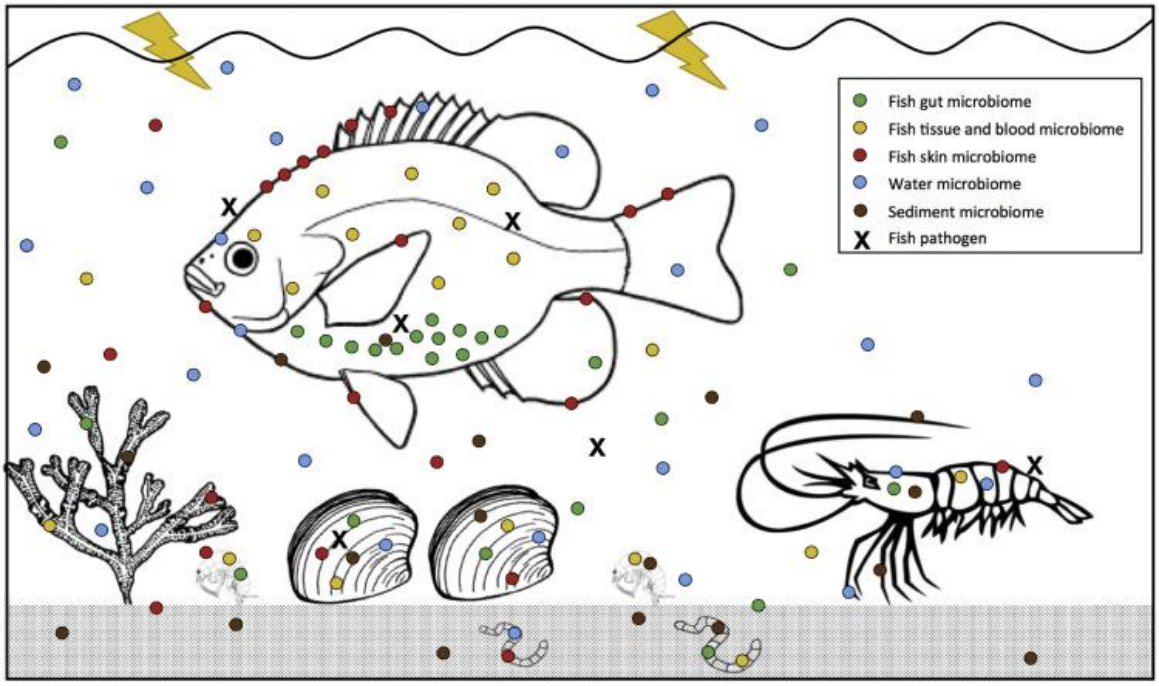


Figure 7: Schematic showing microbiome interactions in an aquatic Host-Symbiont-Environment system. These interactions include the presence of bacteria, viruses, fungi and eukaryotes which can be exchanged between different tissues of various species in the aquatic environment and their surroundings. Figure taken from Bass et al, 2019.

Environmental influencing factors can be further broken down to demonstrate the influence of specific environmental stressors such as increasing sea temperature, hypoxia, and ocean acidification. (Fuhrmann et al, 2019; Khan et al 2018, Liu et al, 2023; 2018; Scanes et al, 2021b; Scanes et al, 2021c; Zhong et al, 2024).

Elevated levels of pCO_2 (a condition of ocean acidification), is a significant driver of changes to microbial diversity and richness within the haemolymph of *Saccostrea glomerata* (the Sydney Rock oyster), causing shifts in low abundance taxa (Scanes et al, 2021b). This may be of particular importance with regards to disease outbreaks, where potentially pathogenic bacteria become more abundant and established within the host. A state of hypoxia (decreased water oxygenation), is also able to influence the bacterial abundance and diversity profiles on the hepatopancreas of *Crassostrea virginica*. Whilst it may be expected that hypoxic stress would result in lower bacterial diversity, the opposite finding was reported, although the physiological effects of this were not tested (Khan et al, 2018).

The effect of temperature on the microbiome is more commonly investigated, with animals being subjected to simulated ocean warming (Scanes et al, 2021c; Zhong et al, 2024) or studied throughout natural summer mortality events (Liu et al, 2023). Importantly, Zhong et

al 2024 describes the effects of rising temperatures on the eukaryotic and prokaryotic communities *within C. gigas* spat. This research reports the variable of temperature to be associated with significant changes to the microbiome. Differences in the relative abundances of core bacterial taxa including members of the Chloroflexota and Bacteroidetes phyla were reported between spat subjected to water at 18°C vs 24°C. The relative abundance of the ciliate *Pseudoperkinsus tapetis* (a pathogenic species in clams), increased within higher temperature groups. This gives weight to the hypothesis that increasing water temperatures may encourage pathogenic microbes and potentially increase mortality rates. Indeed, Scanes et al, 2021c reported an association between elevated water temperature and species richness of *Saccostrea glomerata* haemolymph (although this was not associated with oyster mortality). This description of the microbiome at a prokaryotic and eukaryotic level, (Zhong et al, 2024) is not commonly found within the literature but is greatly needed in order to gain a more holistic understanding of the role and function of the microbiome under these environmental stressors.

Seawater pH, temperature and hypoxia influence the microbiome in a range of different oyster species. The most significant of these factors differs between species and/or studies. However, the microbial changes influenced by these climate change stressors may all increase disease susceptibility. Similar effects have been investigated in viral diseases, with elevated pCO_2 levels increasing the susceptibility of *C. gigas* to OSHV-1 (Fuhrmann et al, 2019). The microbial changes due to these environmental factors may compromise host health and encourage disease outbreaks. This poses a significant threat to both wild oyster populations and their aquaculture.

4.4 From a hatchery perspective:

Understanding possible influencing factors of the oyster microbiome is an important aspect of host health and disease prevention. These influencing factors therefore also likely underpin the success of seedstock production within hatcheries. Within the hatchery environment itself, there are various processes and practices which also have potential to influence the larval microbiome (Figure 8). Oyster hatcheries for example routinely filter seawater, produce microalgae as live feed and select different methods of spawning. In comparison to climate change focused environmental factors, these hatchery-focused practices are under-

researched within the current literature. Understanding the microbial effects of these practices does however have great potential to identify possible sources of microbial contamination and report on the efficiency of current microbial control techniques. This could ultimately allow for fewer larval mortality events and improve the efficiency of seedstock production.

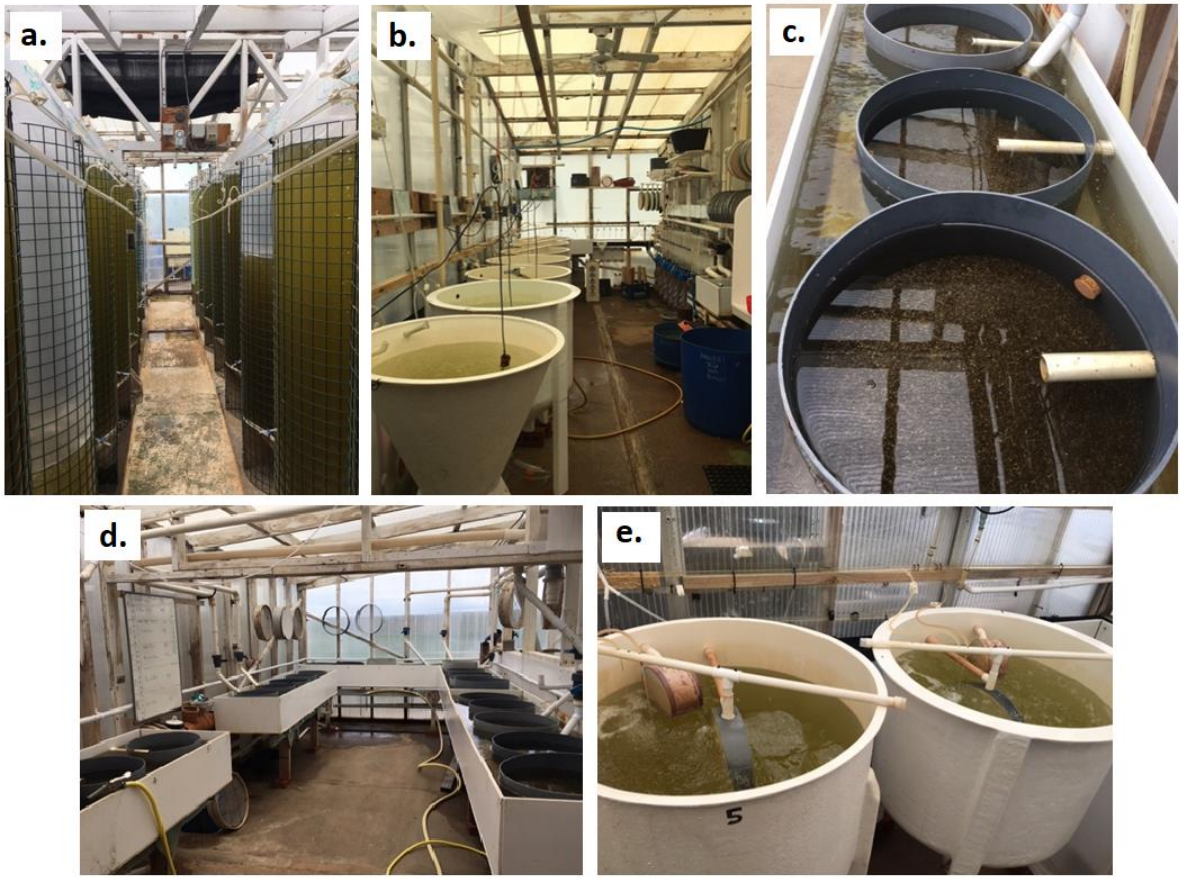


Figure 8: Photographs showing a examples of a commercial hatchery environment including processes of **a.** large scale algal production **b.** larval cultivation **c.** spat maturation in tanks **d.** spat growing room **e.** close up of larval cultivation tanks. Photographs taken at Morecambe Bay oyster hatchery.

Aquaculture system type, flow through (FT) vs recirculating (RS), has little effect on the larval microbiome, with both systems containing similar levels of culturable bacteria and no major bacterial groups being able to differentiate between the two (Asmani et al, 2016). Although microbial differences were not detected between the two systems, microbial variations of larvae and their surrounding seawater were detected within the FT and RS systems over time - mainly characterised in the larvae by a decrease in overall diversity. This finding may provide

evidence of the larval microbiome becoming established over time and favouring specific taxa. The occurrence of microbial variation with time (irrespective of the aquaculture system used) suggests that the developmental stage at which microbial control strategies are administered should be closely considered.

In contrast, the influence of hatchery type (commercial versus research-focused) has been suggested to have an influence over the larval microbiome of *C. virginica* (Arfken et al, 2021). This was attributed to higher relative abundances of the Saprospiraceae and Methylophilaceae families at research-focused hatcheries, and the Mycoplasma, Lactobacillus and Planctomycetaceae families at commercial hatcheries (Arfken et al, 2021). Key differences between these two hatchery types include location, water filtration and feeding methods, which are likely to have driven the microbial differences in the larvae observed between the two hatchery types. It is therefore possible that similar microbial differences may be observed within hatcheries of the same type but which are at different locations or practice different feeding and water filtration methods. Although defining these differences is useful in characterising the influencing factors of the larval microbiome, further research is needed to investigate the functional role of these bacterial families on host health. This would allow for better informed microbial control strategies to be implemented across a range of different hatchery environments.

Algal feed is another key hatchery practice which has the ability to influence larval microbiomes (Arfken et al, 2021; Vignier et al, 2021). The bacterial family Cryomorphaceae, which is most commonly isolated from microalgae has been previously detected in *Crassostrea virginica* larvae (Arfken et al, 2021). In studies with *C. gigas* larvae (Vignier et al, 2021), feeding using stressed microalgae (microalgae with an increased pH) was correlated with deformities, reduced swimming ability and slowed development. Notably, bacterial richness was also found to increase in larvae fed with stressed algae; bacteria belonging to genera of *Halomonas* and *Marinomonas* were particularly prominent and are also closely associated with impaired larval performance (Vignier et al, 2021). The overfeeding of larvae was also correlated with an increase of *Vibrio* within the microbiome – a genera of bacteria commonly known to cause disease outbreak events in oyster hatcheries (Prado et al, 2005; Richards et al, 2015; Ushijima et al. 2018). This study is therefore particularly useful in providing evidence that algal feed is an influencing factor of the larval microbiome, and can

be detrimental to larval health if compromised. Furthermore, reporting an association of particular algal conditions with certain bacterial taxa and larval phenotypes is vital information in order to fully understand the conditions and practices that encourage the outbreak of disease events in hatcheries.

In conclusion, although under researched, there is evidence to suggest that hatchery practices have an influential effect on the oyster larval microbiome. Most notably, these include water treatment, hatchery location (Arfken et al, 2021) and feeding (Arfken et al, 2021; Vignier et al, 2021). Improving our understanding of the link between hatchery techniques and larval microbial communities has the potential to reduce larval mortality events through better targeted control strategies. Future studies would greatly benefit from investigating the phenotypic effects that microbial variations have on oyster larvae. As this research progresses, results could inform hatchery practices for maintaining stable larval microbiomes and reducing bacterial disease outbreaks.

5.0 Manipulation of the microbiome:

5.1 The use of antibiotics within aquaculture:

With disease posing such a significant threat to aquaculture (section 2.1), disease mitigation strategies are one of the most common objectives within current aquaculture research. As is also the case in terrestrial farming, the most widely practiced method of limiting disease events in aquaculture is the use of antibiotics (Adenaya et al, 2023; Cabello, 2006; Sun et al, 2020; Shanmugam et al, 2011). Global antibiotic consumption in aquaculture is estimated to increase by 2030, driven mainly by aquaculture expansion in Asia (Schar et al, 2020); (Figure 9). Widespread use is proliferated by intensive farming practices, driven by an increasing demand for global proteins sources (Adenaya et al, 2023). Antibiotics are defined as chemicals which are designed to inhibit the growth of, or kill pathogenic bacteria (Shanmugam et al, 2011), used mainly to prevent and treat bacterial infections but also sometimes as a growth promotor (Sun et al, 2020). In aquaculture, they are administered via addition to feed, water, or injection (in the case of fish); (Cabello, 2006). Commonly used groups of antibiotics include (but are not limited to) Quinolones, β-Lactams and Chloramphenicols which are used for the prevention of a range of bacterial diseases in fish and shellfish (Sun et al, 2020).

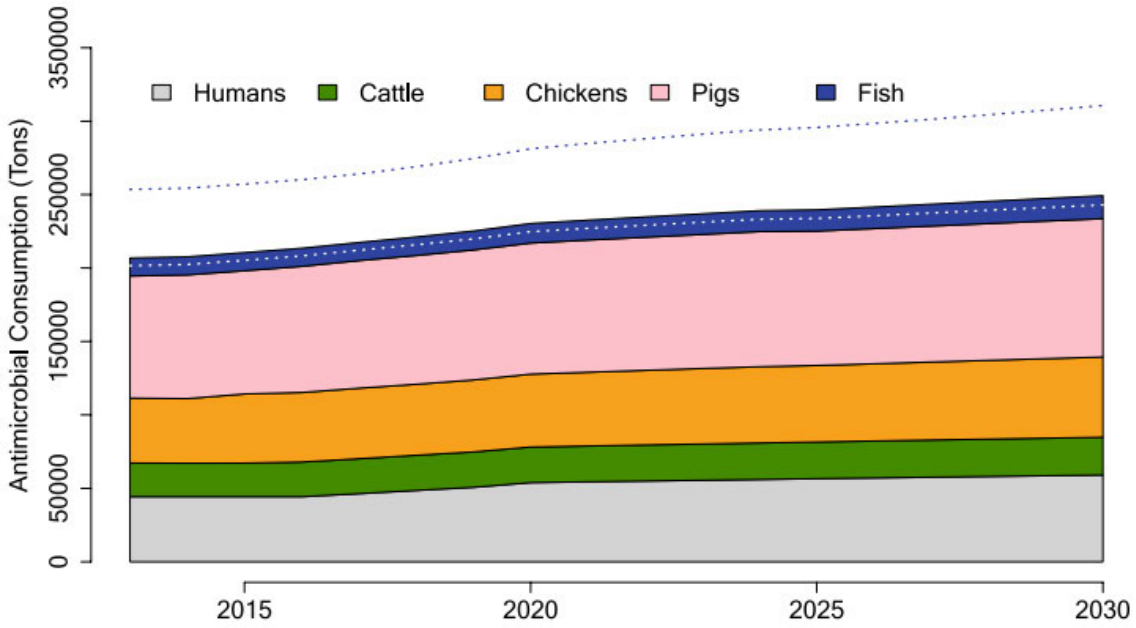


Figure 9: The predicted rise in global antimicrobial consumption between 2013 and 2030. Aquaculture projections include those for fish and not shellfish species. Dotted lines represent the 95% uncertainty interval for fish. Figure taken from Schar et al, 2020.

Despite the successful application of antibiotics, it is well reported upon that their overuse and misuse have a number of detrimental effects which seriously hinder the sustainability of aquaculture and pose significant threats to environmental and consumer health (Adenaya et al, 2023; Cabello, 2006; Sun et al, 2020; Hemamalini et al, 2021). An immediate limitation to the use of antibiotics is the emergence of antibiotic resistant bacteria (AMR) (Sun et al, 2020; Okeke et al, 2022; Geetha Preena et al, 2020). Indeed, significant aquaculture species such as carp, salmon, tilapia and shrimp have all been reported to possess AMR (Geetha Preena et al, 2020). Improper waste disposal from aquaculture sites using antibiotic treatments also allows for accumulation of antibiotic residues in the environment (Le et al, 2023; Hemamalini et al, 2021), with fish aquaculture being the primary source of antibiotics in coastal waters (Adenaya et al, 2023). This can exert a selective pressure which selected for the development of AMR within the aquatic environment as well as having detrimental effects on the growth and reproduction of marine organisms (Maghsodian et al, 2022). The accumulation of antibiotic residues in fish and shellfish products also poses a threat to the health of the consumer (Cabello, 2006), through the development of antimicrobial drug resistance, carcinogenity and the disruption of the normal intestinal microbiome (Okocha et al. 2018).

Contrary to current reviews (Yeh et al, 2020), anecdotal evidence reports that antibiotics are used within oyster aquaculture although under strict regulation. In fact, strains of the bacterial pathogen *Vibrio parahaemolyticus* isolated from *C. gigas* were found to have ampicillin and vancomycin resistance (Kang et al, 2018). Reporting of decreased susceptibility to the antibiotic oxytetracycline in all bivalve species further suggests that antibiotic usage has allowed for the emergence of AMR within oyster aquaculture (Salgueiro et al, 2021). The development of such AMR within oyster aquaculture is likely influenced by a variety of factors such as oyster species, developmental stage, hatchery location and season (Salgueiro et al, 2021). The emergence of antimicrobial resistance therefore involves a complex network of interactions which requires oyster hatchery-based scenarios to be considered on an individual basis.

The limitations of antibiotic usage within aquaculture are varied and widespread causing significant implications to consumer and environmental health and well as the sustainability of the industry. Decreasing the use of antibiotics and adhering to regulation is the most effective way of reducing these negative implications (Sun et al, 2020). This is however dependent upon the development of alternative methods to treat the large-scale disease outbreaks which so greatly threated the expansion of aquaculture (Sun et al, 2020). This therefore creates a significant area for future research, where the development of antibiotic alternatives has great potential to reduce disease within aquaculture and secure the sustainability of the industry.

5.2 Alternative treatments to antibiotics in oyster aquaculture:

Alternative methods to antibiotic usage are being more frequently researched for use within a range of different aquaculture species. These have been developed to varying extents with the most commonly used including vaccines (Dadar et al, 2017; Gudding and Van Muiswinkel, 2013; Sommerset et al, 2005) and probiotics (Gatesoupe,1999; Hai et al, 2015; Martínez Cruz et al, 2012; Ringø et al, 2020; Yeh et al, 2020). Other methods such as prebiotics (Carbone and Faggio, 2016; Ringø et al, 2010; Wee et al, 2024) and bacteriophages (Nachimuthu et al, 2021; Ninawe et al, 2020; Schulz et al, 2022) are less well developed but still provide promising alternatives with potential to improve the sustainability and security of oyster aquaculture.

Probiotics are the most commonly used alternative treatment to antibiotics (Gatesoupe, 1999), although their application to the aquaculture industry lags behind that of terrestrial farming. In fact, the first probiotic treatments trailed in fish were prepared from commercial probiotic treatments given to land animals (Gatesoupe, 1999). The use of probiotics within aquaculture has developed greatly since the first known trails (Kozasa, 1986), with an increasing number of probiotic candidates, target pathogens and host species being investigated. This development has however been more focused towards the improvement of fish aquaculture. In comparison to fish species, far fewer publications have investigated probiotic applications to shellfish (Figure 10), where publication rate data is exclusive to shrimp species. The potential of probiotic use within bivalve hatcheries are therefore even less well investigated, creating a significant knowledge gap within the current literature.

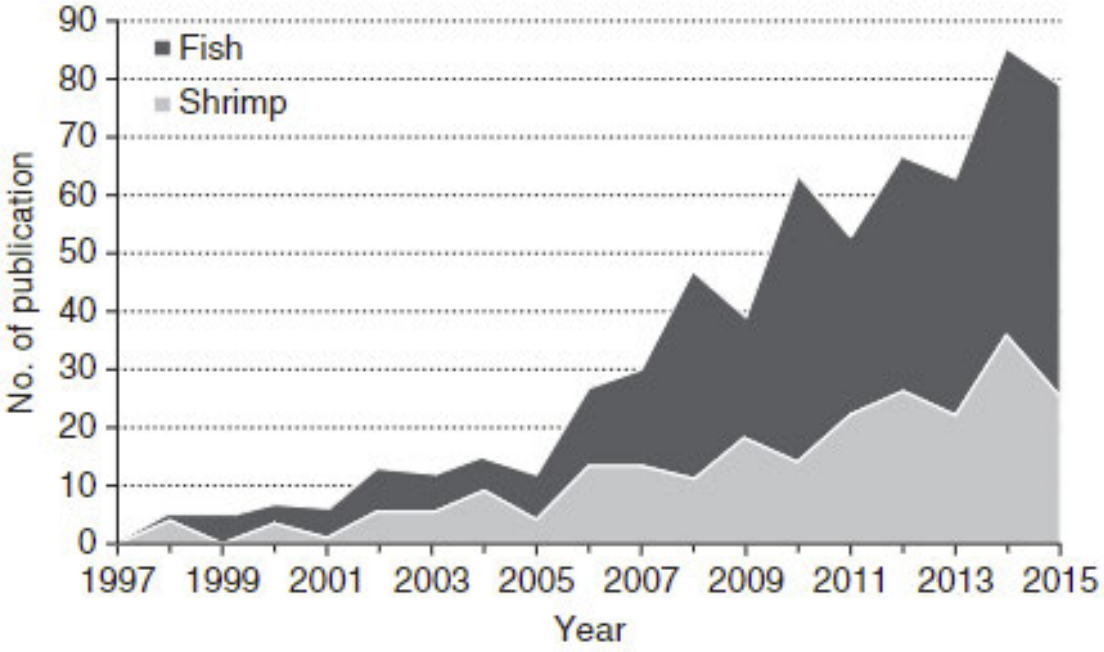


Figure 10: Number of publications between 1997 and 2015 which investigate probiotic treatments for fish and shrimp species. Figure taken from: Sharifuzzaman and Austin 2017.

Since the original definition (Parker, 1974), the term 'probiotic' has been continuously redefined. They are however now generally accepted as 'live and/or dead microbial feed supplements or water additives which are administered to improve the rearing water quality or enhance the physiological and immune responses of aquatic animals and reduce the use of chemicals and antibiotics in aquaculture' (Hai, 2015). The development of a probiotic

treatment begins with the isolation of a bacterial strain either directly from the host itself (often the GI tract) or its environment (surrounding seawater or marine sediment), (Hai et al, 2015; Yeh et al, 2020). An ideal probiotic candidate has no detrimental effect to host health, is able to colonise tissues of interest and possess no virulent or antibiotic resistant genes (Hai et al, 2015). Knowing that probiotic effects cannot always be observed in vitro, the suspected probiotic strain is then tested for its prohibitory effects against the target pathogen *in vivo* (Hai et al, 2015).

The transfer of probiotic treatments from fish to shellfish aquaculture has been slowed by the lengthy processes needed to gain FDA approval and sponsorship for commercial-scale development (Yeh et al, 2020). Despite this, current research has highlighted promising probiotic candidates which have the potential to reduce disease mortality rates and improve the sustainability of shellfish farming. The isolation of *Thalassobacter utilis* is one of the first probiotic candidates identified for shellfish production (Maeda and Liao, 1992). The addition of this bacteria improves larval survival of giant tiger prawn and swimming crab larvae through providing inhibitory effects against the bacterial pathogen *Vibrio anguillarum* (Meada ad Liao, 1992).

In relation to inhibiting specific pathogens, strains belonging to the *Phaeobacter* (Karim et al, 2013), and lactic acid bacterial groups (Kang et al, 2018) are able to confer resistance against *Vibrio tubiashii*, *Roseovarius Crassostrea (*Karim et al, 2013)*, Vibrio parahaemolyticus*, *Streptococcus iniae* and *Edwardsiella tarda* (Kang et al, 2018). Regardless of stressors placed on the host from pathogenic bacteria, probiotic treatments can also be beneficial to oyster growth, with strains of *Bacillus* bacteria improving the growth and survival rate of *Crassostrea skiamea* spat (Sánchez-Ortiz et al, 2020). These studies provide promising evidence for the potential of probiotics to reduce the impact of disease in shellfish aquaculture, highlighting the need to develop these strategies further, and replicate current results in extensive scale commercial farms (Amenyogbe et al, 2020). Through preliminary experiments, this thesis aims to address this need for further development of probiotics strategies in shellfish through the screening of candidate Rhodobacteraceae strains for the protection of *C. gigas* against *V. aestuarianus* and *V. splendidus* infection (see Chapter five).

The development of prebiotic treatments alongside probiotics, has allowed for another possible alternative to antibiotic usage (Ganguly et al, 2010; Noman et al, 2024; Ringø et al,

2010). Prebiotic treatments are defined as any compound which serves as a source of nutrients for beneficial microorganisms which reside within the host. (Wee et al, 2024). By promoting beneficial microorganisms, this can have a number of positive effects such as increased growth rates, enhancement of the immune system and reduced stress in the host (Wee et al, 2024). All of these effects are highly beneficial to the host, likely reducing disease as well as improving the welfare of the animal. Recent reviews highlight the development of prebiotics within shellfish aquaculture to be limited to applications in Pacific Whiteleg shrimp cultivation (Arun et al, 2023; Wee et al, 2024). This is again, another example of current methods being less well developed for applications to oyster cultivation.

Vaccines offer another alternative approach to antibiotics and are being increasingly well developed. Whilst this is a huge advancement for aquaculture as a whole, vaccination is not feasible for the treatment of shellfish due to their lack of adaptive immunity. The concept of immune priming does however offer a promising alternative for shellfish, with applications of increased resistance to OsHV-1 (de Kantzow et al, 2023). In fish aquaculture, vaccines are one of the most cost-effective and efficient ways of reducing diseases such as yersioniosis and vibriosis in farmed fish (Mondal and Thomas, 2020). For instance, vaccination strategies in Norway have been instrumental in reducing the use of antibiotics on fish farms (Figure 11). Here, the implementation of vaccinations allowed for reduced used of antibiotics. Although not directly applicable to shellfish, evidence of successful vaccination programmes highlight the advantage of disease prevention over cure - a concept which shellfish aquaculture would also greatly benefit from.

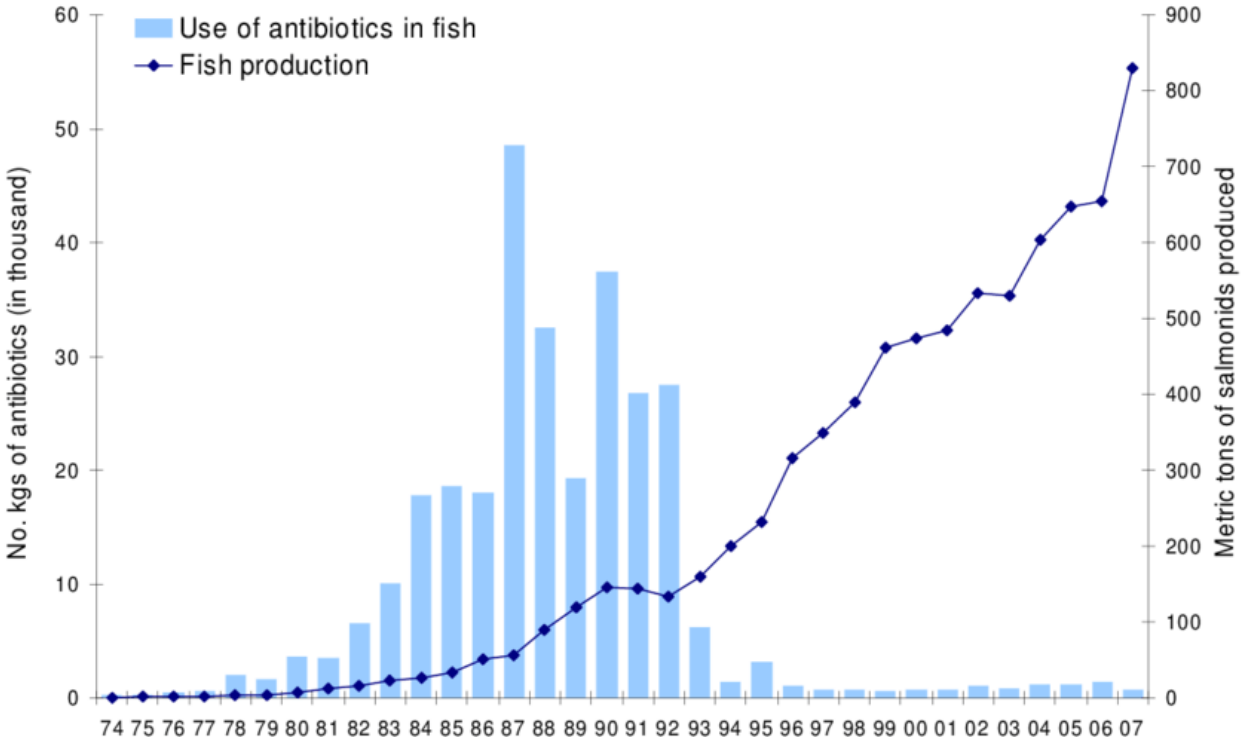


Figure 11: Trends in antibiotic usage and fish production in Norway from 1974 to 2008. Figure taken from Benzie, 2010.

Another possible alternative to the use of antibiotics includes the development of bacteriophage-based treatments (Ninawe et al, 2020; Madhusudana Rao and Lalitha, 2015; Richards, 2014; Sieiro et al 2020; Yang et al 2024). Bacteriophages are viruses which have the capacity to destroy target bacteria through lytic and lysogenic infection cycles (Oliveira et al, 2012). These treatments are currently utilised in aquaculture, with the most recent review reporting 24 different treatments (Sieiro et al, 2020), three of which are designed specifically for use in oyster species. These treatments are based on the *Siphoviridae* and N4-like podovirus families for the treatment of *Vibrio parahaemolyticucs* (Jun et al, 2014), *Vibrio coralliilyticucs* (Kim et al, 2019) and *Vibrio splendidus* (Katharios et al 2017) in oyster cultivation (Sieiro et al, 2020). The reduction of these key pathogens without using antibiotics makes bacteriophage treatment a promising strategy in the development of oyster aquaculture. They are however limited by unknown effects on the host and environmental communities, gene transfer and the effects of organic matter released after bacteriophage lysis (Culot et al, 2019; Yang et al, 2024). Addressing these knowledge gaps would allow for

such long-term limitations to be investigated, further improving the efficacy and sustainability of this already promising treatment.

6.0 Summary and future perspectives:

Aquaculture has allowed for increased protein production demanded from a growing global population. Despite its rapid growth since the 1950's, limitations to aquaculture prevent its further expansion and threaten the sustainability of the industry. The most notable of these limitations are the occurrence of disease events which threaten the production of finfish, crustacean and molluscan species. Disease events in aquaculture case huge economic loses and are often multi-pathogenic, involving complex interactions between pathogens, host health and its environment. The microbiome of any organism is well understood to play a key role in host health. As sessile filter feeding organisms that are extrinsically linked to their environment, the microbes that reside within oysters are also impart control over host health and crucially, disease susceptibility. This review collates information from current research to discuss findings of the core taxa (see section 3.3), pathogenic influences (see section 3.4), source (see section 4.1), and influencing factors (see sections 4.2, 4.3 and 4.4), of the microbiome in a range of different oyster species. Synthesising this information is vital in order to highlight the importance of the oyster microbiome and explore microbiome manipulation strategies (see section 5), for the improvement of larval health and development of sustainable disease mitigation strategies. Microbial research has great potential to improve the ways in which oysters are produced, with future research in this area having relevant applications to both commercial sand wild settings.

Important knowledge gaps have been identified throughout this literature review. Addressing the functional roles of non-pathogenic bacteria within the oyster microbiome could be of great advantage. This could provide important information about oyster health and immune function, ultimately allowing for improved disease control and prevention strategies. The identification of the core larval microbiome is especially relevant to fulfilling this knowledge gap, since non-transient taxa are most likely to play functional roles in biological processes such as homeostasis or development (Arfken et al, 2021). This thesis contributes to this knowledge gap through the identification of *O. edulis* and *C. gigas* core taxa at UK hatcheries

(see Chapter three). Future research may build upon this knowledge by using the core taxa identified throughout chapter three for gene annotation, transcriptomics or metabolomics analysis to infer their functional roles.

The influence that the external environment imparts on the oyster microbiome has been well discussed (see section 4.3). Despite this, the majority of research is often limited to comparing the microbiome of oysters to that only of their culture water or algal feed (Arfken et al, 2021; Laroche et al, 2018; Vignier et al, 2021). A commercial hatchery environment however involves a far wider range of microbial influencing factors – all of which could provide key information about microbial sources and contamination variables, as well as providing a route to manipulation. Future research is therefore needed to examine the oyster microbiome in relation to the hatchery environment and processes. Similarly, this knowledge gap is also addressed by chapter three, where the microbial compositions of larvae and their culture water are reported against compositions of sea water throughout the filtration process, biofilm and algae at two different UK hatcheries.

This review highlights another key knowledge gap in the need to develop alternative methods to antibiotics as a means microbial manipulation (see section 5.1 and 5.2). More specifically, the role of algal feeds has been identified as under researched yet important influencing factor of the larval microbiome (see section 4.4). Additional research in this area holds promise for the development of new microbiome manipulation methods, where certain algal species may prove beneficial through the promotion of certain bacterial taxa or physiochemical water parameters. Chapter four of this thesis aims to address this knowledge gap through the investigating the use of Tetraselmis suecica as a mean of microbial manipulation.

Recommendations for future research share a common theme of practical applications to the hatchery environment - prioritising either the general improvement of larval health or development of disease mitigation strategies. This is ultimately with the end objective of improving the ways in which oysters are produced and contributing to food sustainability and ecological restoration efforts.

Project aims and Chapter-specific Objectives:

The previous section of this thesis highlights the important role of the oyster microbiome in host health and the ability of microbial research to improve oyster health and contribute to food sustainability goals. In order to fully utilise the potential of microbial research, there is a need to expand studies of the baseline larval microbiome, improve understanding of interactions between larvae and the hatchery environment and develop alternative methods of microbial manipulation to prevent the use of antibiotics. The following chapters of this thesis aim to aid in resolving these knowledge gaps, incorporating microbial studies of both *C. gigas* and *O. edulis*, in both wild and farmed settings.

Chapter two: To provide baseline data to describe the first investigation of the environmental microbiome throughout a wild bivalve mollusc spawning event. This study includes the use of full-length 16S sequencing (as is also used throughout chapters three and four), for the collection of bacterial identification and abundance data. This baseline data is the first of its kind for any natural oyster spawning event and allows for recommendations for future studies to improve cultivation strategies for *O. edulis*.

Chapter three: To collect broad-scale microbial data during spawning events of *C. gigas* and *O. edulis* from two UK oyster hatcheries (Morecambe Bay commercial hatchery, and Portsmouth University research hatchery). This broad-scale data was directed through three key aims:

1. To identify core larval taxa and how these change throughout the rearing process.
2. Compare bacterial profiles of larvae to those of their hatchery environment.
3. Investigate how hatchery processes specific to these locations including water filtration and algal cultivation methods influence microbial communities.

This microbial data aimed to improve the current understanding of the oyster microbiome and hatchery environment within commercial-scale production.

Chapter four: In the light of increased antibiotic usage within aquaculture, there is an unquestionable need to develop alternative methods of microbial manipulation. This chapter therefore aims to investigate methods of temperature and diet control as methods of microbial manipulation in *C. gigas* larvae using both culture dependent and independent techniques.

Chapter five: This chapter aims to investigate another alternative method of microbial manipulation, with the screening of *Rhodobacteraceae* strains for their potential inhibitory effects against the key oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. In vivo experiments using agar plate assays aimed to select of suitable *Rhodobacteraceae* strains to be used for in vivo bath challenged adult *C. gigas* individuals.

Chapter six: Presents the general discussion, main conclusions and future directions of the thesis.

References:

Adenaya, A., Berger, M., Brinkhoff, T., Ribas-Ribas, M., & Wurl, O. (2023). Usage of antibiotics in aquaculture and the impact on coastal waters. *Marine Pollution Bulletin*, 188. https://doi.org/10.1016/j.marpolbul.2023.114645

Adewolu, M. A., Akintola, S. L., Jimoh, A. A., Owodehinde, F. G., Whenu, O. O., & Fakoya, K. A. (2009). Environmental threats to the development of aquaculture in Lagos State, Nigeria. *Eur J Sci Res*, 34(3), 337-347.

Amenyogbe, E., Chen, G., Wang, Z., Huang, J., Huang, B., & Li, H. (2020). The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions.: a review. *Aquaculture International*, 28, 1017-1041.

Arfken, A., Song, B., Allen, S. K., & Carnegie, R. B. (2021). Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. *Aquaculture*, 531. https://doi.org/10.1016/j.aquaculture.2020.735955

Arun, D., Midhun, S. J., Sheeja, C. C., Maurya, A. K., & Divya, L. (2023). Probiotics and prebiotics in aquaculture. In *Recent Advances in Aquaculture Microbial Technology*, 209–226. Elsevier. https://doi.org/10.1016/B978-0-323-90261-8.00005-5

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. 2016. Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*. 464, 434–444. https://doi.org/10.1016/j.aquaculture.2016.07.020

Akter, S., Wos-Oxley, M. L., Catalano, S. R., Hassan, M. M., Li, X., Oxley, A. P. A., & Qin, J. G. (2024). Dietary regulation of gut bacteria in the Pacific oyster *Magallana gigas* and Mediterranean mussel *Mytilus galloprovincialis*. *Aquaculture and Fisheries*. https://doi.org/10.1016/j.aaf.2024.08.001

Benzie, J. (2010). Current Status and Options for Biotechnologies in Fisheries and Aquaculture in Developing Countries E FAO International Technical Conference Agricultural biotechnologies in developing countries: Options and opportunities in crops, forestry, livestock, fisheries and agro-industry to face the challenges of food insecurity and climate change (ABDC-10) Current Status and Options for Biotechnologies in Fisheries and Aquaculture in Developing Countries.

Bass, D., Stentiford, G. D., Wang, H. C., Koskella, B., & Tyler, C. R. (2019). The Pathobiome in Animal and Plant Diseases. *Trends in Ecology and Evolution*. Elsevier Ltd. https://doi.org/10.1016/j.tree.2019.07.012

Bean, T. P., Farley, H., Nascimento-Schulze, J., & Regan, T. (2024). Scottish oyster mortality event and association with *Vibrio aestuarianus*. *Aquaculture Reports*, 39, 102480.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology*, 132(3-4 SPEC. ISS.), 249–272. https://doi.org/10.1016/j.vetpar.2005.07.005

Botta, R., Asche, F., Borsum, J. S., & Camp, E. V. (2020). A review of global oyster aquaculture production and consumption. *Marine Policy*, 117. https://doi.org/10.1016/j.marpol.2020.103952

Boudry, P., Allal, F., Aslam, M. L., Bargelloni, L., Bean, T. P., Brard-Fudulea, S., Brieuc, M. S. O., Calboli, F. C. F., Gilbey, J., Haffray, P., Lamy, J. B., Morvezen, R., Purcell, C., Prodöhl, P. A., Vandeputte, M., Waldbieser, G. C., Sonesson, A. K., & Houston, R. D. (2021). Current status and potential of genomic selection to improve selective breeding in the main aquaculture species of International Council for the Exploration of the Sea (ICES) member countries. In *Aquaculture Reports*, 20. Elsevier B.V. https://doi.org/10.1016/j.aqrep.2021.100700

Bui, S., Oppedal, F., Sievers, M., & Dempster, T. (2019). Behaviour in the toolbox to outsmart parasites and improve fish welfare in aquaculture. *Reviews in Aquaculture*, 11(1), 168–186. https://doi.org/10.1111/raq.12232

Bushek, D., & Ford, S. E. (2016). Anthropogenic impacts on an oyster metapopulation: Pathogen introduction, Climate change and responses to natural selection. *Elementa*, 2016, 1–13. https://doi.org/10.12952/journal.elementa.000119

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. In *Environmental Microbiology* 8 (7), 1137–1144. https://doi.org/10.1111/j.1462-2920.2006.01054.x

Capone, R & Di Giulio. (2001). Urbanisation of Coastal in Mediterranean Areas and Renewable Natural Resources. *Medit.* 12 (2), 30-37.

Carbone, D., & Faggio, C. (2016). Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. In *Fish and Shellfish Immunology*. 54, 172–178. https://doi.org/10.1016/j.fsi.2016.04.011

Cho, A., Finke, J. F., Zhong, K. X., Chan, A. M., Saunders, R., Schulze, A., Warne, S., Miller, K. M., & Suttle, C. A. (2024). The core microbiome of cultured Pacific oyster spat is affected by age but not mortality. *Microbiology Spectrum*. https://doi.org/10.1128/spectrum.00031-24

Clerissi, C., de Lorgeril, J., Petton, B., Lucasson, A., Escoubas, J. M., Gueguen, Y., Toulza, E. (2020). Microbiota Composition and Evenness Predict Survival Rate of Oysters Confronted to Pacific Oyster Mortality Syndrome. *Frontiers in Microbiology*, 11, 1–11. https://doi.org/10.3389/fmicb.2020.00311

Clifford, R. J., Milillo, M., Prestwood, J., Quintero, R., Zurawski, D. V., Kwak, Y. I., Mc Gann, P. (2012). Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR. *PloS One*, 7(11), 7–12. https://doi.org/10.1371/journal.pone.0048558

Cole, D. W., Cole, R., Gaydos, S. J., Gray, J., Hyland, G., Jacques, M. L., & Au, W. W. (2009). Aquaculture: Environmental, toxicological, and health issues. *International journal of hygiene and environmental health*, 212(4), 369-377.

Colsoul, B., Boudry, P., Pérez-Parallé, M. L., Bratoš Cetinić, A., Hugh-Jones, T., Arzul, I., ... & Pogoda, B. (2021). Sustainable large-scale production of European flat oyster (*Ostrea edulis*)

seed for ecological restoration and aquaculture: a review. *Reviews in Aquaculture*, 13(3), 1423-1468.

Colwell, R, R & Liston, J. (1959). Bacteriological Study of the Natural Flora of Pacific Oysters (*Crassostrea gigas*). Microbiology of Shellfis*h*, (8) 104 – 109.

Cram, J. A., McCarty, A. J., Willey, S. M., & Alexander, S. T. (2024). Microbial community structure variability over the development of healthy and underperforming oyster larval hatchery broods. *Frontiers in Aquaculture*, 3, 1427405.

Culot, A., Grosset, N., & Gautier, M. (2019). Overcoming the challenges of phage therapy for industrial aquaculture: A review. In *Aquaculture*, 513. Elsevier B.V. https://doi.org/10.1016/j.aquaculture.2019.734423

Dadar, M., Dhama, K., Vakharia, V. N., Hoseinifar, S. H., Karthik, K., Tiwari, R., Khandia, R., Munjal, A., Salgado-Miranda, C., & Joshi, S. K. (2017). Advances in Aquaculture Vaccines Against Fish Pathogens: Global Status and Current Trends. In *Reviews in Fisheries Science and Aquaculture*. 25 (3), 184–217. https://doi.org/10.1080/23308249.2016.1261277

Dai, W., Ye, J., Liu, S., Chang, G., Xu, H., Lin, Z., & Xue, Q. (2022). Bacterial community dynamics in Kumamoto oyster *Crassostrea sikamea* hatchery during larval development. *Frontiers in Microbiology*, 13, 933941.

De Decker, S., & Saulnier, D. (2011). Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: Evidence of early infection and down-expression of immune-related genes. *Fish and Shellfish Immunology*, 30(2), 691–699. https://doi.org/10.1016/j.fsi.2010.12.017

de Kantzow, M., Hick, P. M., & Whittington, R. J. (2023). Immune Priming of Pacific oysters (*Crassostrea gigas*) to Induce Resistance to Ostreid herpesvirus 1: comparison of infectious and inactivated OsHV-1 with poly I: C. *Viruses*, 15(9), 1943.

Delmotte, J., Chaparro, C., Galinier, R., de Lorgeril, J., Petton, B., Stenger, P. L., Vidal-Dupiol, J., Destoumieux-Garzon, D., Gueguen, Y., Montagnani, C., Escoubas, J. M., & Mitta, G. (2020). Contribution of Viral Genomic Diversity to Oyster Susceptibility in the Pacific Oyster Mortality Syndrome. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.01579

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J. M., Haffner, P., Dégremont, L., Charrière, G. M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., … Mitta, G. (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06659-3

De Silva, S.S. and Soto, D. (2009). Climate change and aquaculture: potential impacts, adaptation and mitigation. In K. Cochrane, C. De Young, D. Soto and T. Bahri (eds). Climate change implications for fisheries and aquaculture: overview of current scientific knowledge. FAO Fisheries and Aquaculture Technical Paper. No. 530. Rome, FAO, 151-212.

Dégremont, L. (2011). Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. *Aquaculture*, 317(1–4), 94–98. https://doi.org/10.1016/j.aquaculture.2011.04.029

Dégremont, L., Nourry, M., & Maurouard, E. (2015). Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: Response to selection after four generations. *Aquaculture*, 446, 111–121. https://doi.org/10.1016/j.aquaculture.2015.04.029

Douillet, P., & Langdon, C. J. (1993). Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. *Biological Bulletin*, *184*(1), 36–51. https://doi.org/10.2307/1542378

Dubé, C. E., Ky, C. L., & Planes, S. (2019). Microbiome of the black-lipped pearl oyster pinctada margaritifera, a multi-tissue description with functional profiling. *Frontiers in Microbiology*, 10(JULY). https://doi.org/10.3389/fmicb.2019.01548

Dubert, J., Barja, J. L., & Romalde, J. L. (2017). New insights into pathogenic vibrios affecting bivalves in hatcheries: Present and future prospects. *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2017.00762

Dupont, S., Lokmer, A., Corre, E., Auguet, J.-C., Petton, B., Toulza, E., … Escoubas, J.-M. (2020). Oyster hemolymph is a complex and dynamic ecosystem hosting bacteria, protists and viruses. *Animal Microbiome*, 2(1). https://doi.org/10.1186/s42523-020-00032-w

Elston, R. A. (1993). Infectious diseases of the Pacific oyster, *Crassostrea gigas*. *Annual Review of Fish Diseases*, 3, 259–276. https://doi.org/10.1016/0959-8030(93)90038-D

English, M. K., Langdon, C. J., Schubiger, C. B., & Mueller, R. S. (2023). Dominant bacterial taxa drive microbiome differences of juvenile Pacific oysters of the same age and variable sizes. *Frontiers in Microbiomes*, 2. https://doi.org/10.3389/frmbi.2023.1071186

Ezgeta Balic, D., Radonic, I., Bojanic Varezic, D., Zorica, B., Arapov, J., Staglicic, N., ... & Segvic-Bubic, T. (2020). Reproductive cycle of a non-native oyster, *Crassostrea gigas*, in the Adriatic Sea. *Mediterranean Marine Science*, 21(1), 146-156.

Fan, L., Reynolds, D., Liu, M., Stark, M., Kjelleberg, S., Webster, N. S., & Thomas, T. (2012). Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, 109(27). https://doi.org/10.1073/pnas.1203287109

FAO. 2018. The State of World Fisheries and Aquaculture 2018. Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO

FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome.https://doi.org/10.4060/ca9229en

FAO. 2024. In Brief to The State of World Fisheries and Aquaculture 2024. Blue

Transformation in action. Rome. https://doi.org/10.4060/cd0690en

Farley, C, A., Banfield, W, G., Kasnic, G, Jr., & Foster, W, S. (1972). Oyster Herpes-Type Virus. *Science*, 178 (4062). https://www.science.org/doi/10.1126/science.178.4062.759

Ferreira, J. G., Hawkins, A. J. S., & Bricker, S. B. (2007). Management of productivity, environmental effects and profitability of shellfish aquaculture - the Farm Aquaculture Resource Management (FARM) model. *Aquaculture*, 264(1–4), 160–174. https://doi.org/10.1016/j.aquaculture.2006.12.017

Fraune, S., & Bosch, T. C. G. (2007). Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. *Proceedings of the National Academy of Sciences of the United States of America*, 104(32), 13146–13151. https://doi.org/10.1073/pnas.0703375104

Fu, Q., Yu, Z., Zhao, J., Gao, L., Kong, N., Wang, L., & Song, L. (2024). Spatial compartmentalization and temporal stability of associated microbiota in Pacific oyster *Crassostrea gigas*. *Journal of Oceanology and Limnology*, 42(4), 1348–1358. https://doi.org/10.1007/s00343-024-3189-7

Fuhrmann, M., Richard, G., Quéré, C., Petton, B., & Pernet, F. (2019). Low pH reduced survival of the oyster *Crassostrea gigas* exposed to the Ostreid herpesvirus 1 by altering the metabolic response of the host. *Aquaculture*, *503*, 167–174. https://doi.org/10.1016/j.aquaculture.2018.12.052

Gagnaire, B., Gay, M., Huvet, A., Daniel, J. Y., Saulnier, D., & Renault, T. (2007). Combination of a pesticide exposure and a bacterial challenge: In vivo effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg). *Aquatic Toxicology*, 84(1), 92–102. https://doi.org/10.1016/j.aquatox.2007.06.002

Galtsoff, P, S. (1964). The American Oyster *Crassostrea virginica gmelin*. *Fishery Bulletin*, 64 (1), 355- 381.

Ganguly, S., Paul, I., & Kumar Mukhopadhayay, S. (2010). Application and Effectiveness of Immunostimulants, Probiotics, and Prebiotics in Aquaculture: A Review. In *The Israeli Journal of Aquaculture-Bamidgeh*, 62(3). http://www.siamb.org.il

Gao, Y. M., Zou, K. S., Zhou, L., Huang, X. De, Li, Y. Y., Gao, X. Y., … Zhang, X. Y. (2020). Deep insights into gut microbiota in four carnivorous coral reef fishes from the South China Sea. *Microorganisms*, 8(3). https://doi.org/10.3390/microorganisms8030426

Garlock, T., Asche, F., Anderson, J., Bjørndal, T., Kumar, G., Lorenzen, K., … Tveterås, R. (2020). A Global Blue Revolution: Aquaculture Growth Across Regions, Species, and Countries. *Reviews in Fisheries Science and Aquaculture*, 28(1), 107–116. https://doi.org/10.1080/23308249.2019.1678111

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. In *Aquaculture*, 180. www.elsevier.nlrlocateraqua-online

Geetha Preena, P., Raja Swaminathan, T., Jayadradhan Rejish Kumar, V., & Sarojini Bright Singh, I. (n.d.). *Antimicrobial resistance in aquaculture: a crisis for concern*. https://doi.org/10.2478/s11756-020-00456-4/Published

Genard, B., Pernet, F., Lemarchand, K., Boudry, P., Moraga, D., & Tremblay, R. (2011). Physiological and biochemical changes associated with massive mortality events occurring in larvae of American oyster (*Crassostrea virginica*). *Aquatic Living Resources*, 24(3), 247-260.

Gillingham, M. A., Prüter, H., Montero, B. K., & Kempenaers, B. (2024). The costs and benefits of a dynamic host microbiome. *Trends in Ecology & Evolution*.

Gray, M. W., Alexander, S. T., Beal, B. F., Bliss, T., Burge, C. A., Cram, J. A., … Wilbur, A. E. (2022). Hatchery crashes among shellfish research hatcheries along the Atlantic coast of the United States: A case study at Horn Point Laboratory oyster research hatchery. *Aquaculture*, 546, 737259. https://doi.org/10.1016/j.aquaculture.2021.737259

Green, T. J., Siboni, N., King, W. L., Labbate, M., Seymour, J. R., & Raftos, D. (2019). Simulated Marine Heat Wave Alters Abundance and Structure of *Vibrio* Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event. *Microbial Ecology*. 77, 3. https://doi.org/10.1007/s00248-018-1242-9

Gudding, R., & Van Muiswinkel, W. B. (2013). A history of fish vaccination: Science-based disease prevention in aquaculture. In *Fish and Shellfish Immunology*. 35 (6), 1683–1688. Academic Press. https://doi.org/10.1016/j.fsi.2013.09.031

Gundappa, M.K., Peñaloza, C., Regan, T., Boutet, I., Tanguy, A., Houston, R.D., Bean, T.P. and Macqueen, D.J. (2022). Chromosome-level reference genome for European flat oyster (*Ostrea edulis L*.). *Evolutionary applications*, 15(11), pp.1713-1729.

Hai, N. V. (2015). The use of probiotics in aquaculture. In *Journal of Applied Microbiology*. 119 (4), 917–935. https://doi.org/10.1111/jam.12886

Hadziavdic, K., Lekang, K., Lanzen, A., Jonassen, I., Thompson, E. M., & Troedsson, C. (2014). Characterization of the 18s rRNA gene for designing universal eukaryote specific primers. *PLoS ONE*, 9(2). https://doi.org/10.1371/journal.pone.0087624

Helmer, L., Farrell, P., Hendy, I., Harding, S., Robertson, M., & Preston, J. (2019). Active management is required to turn the tide for depleted *Ostrea edulis* stocks from the effects of overfishing, disease and invasive species. *PeerJ*, 7, e6431.

Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., & Tiwari, B. (2017). Future protein supply and demand: Strategies and factors influencing a sustainable equilibrium. *Foods*, 6(7), 1–21. https://doi.org/10.3390/foods6070053

Hemamalini, N., Shanmugam, S. A., Kathirvelpandian, A., Deepak, A., Kaliyamurthi, V., & Suresh, E. (2022). A critical review on the antimicrobial resistance, antibiotic residue and metagenomics-assisted antimicrobial resistance gene detection in freshwater aquaculture environment. *Aquaculture Research*. 53 (2), 344–366. https://doi.org/10.1111/are.15601

Herbert, R. J. H., Humphreys, J., Davies, C. J., Roberts, C., Fletcher, S., & Crowe, T. P. (2016). Ecological impacts of non-native Pacific oysters (*Crassostrea gigas*) and management measures for protected areas in Europe. *Biodiversity and Conservation*, 25(14), 2835–2865. https://doi.org/10.1007/s10531-016-1209-4

Houston, R. D., Bean, T. P., Macqueen, D. J., Gundappa, M. K., Jin, Y. H., Jenkins, T. L., … Robledo, D. (2020). Harnessing genomics to fast-track genetic improvement in aquaculture. *Nature Reviews Genetics*, 21(7), 389–409. https://doi.org/10.1038/s41576-020-0227-y

Huang, Y. T., Liu, P. Y., & Shih, P. W. (2021). Homopolish: a method for the removal of systematic errors in nanopore sequencing by homologous polishing. *Genome Biology*, 22(1). https://doi.org/10.1186/s13059-021-02282-6

Humphries, F., Benzie, J. A. H., Lawson, C., & Morrison, C. (2021). A review of access and benefit-sharing measures and literature in key aquaculture-producing countries. *Reviews in Aquaculture*, 1–18. https://doi.org/10.1111/raq.12532

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2020). Microbiome diversity and dysbiosis in aquaculture. *Reviews in Aquaculture*. Wiley-Blackwell. https://doi.org/10.1111/raq.12513

Jennings, S., Stentiford, G. D., Leocadio, A. M., Jeffery, K. R., Metcalfe, J. D., Katsiadaki, I., … Verner-Jeffreys, D. W. (2016). Aquatic food security: insights into challenges and solutions from an analysis of interactions between fisheries, aquaculture, food safety, human health, fish and human welfare, economy and environment. *Fish and Fisheries*, 17(4), 893–938. https://doi.org/10.1111/faf.12152

Jones, J. B. (2006). Why won't they grow? - Inhibitory substances and mollusc hatcheries. *Aquaculture International*, 14(4), 395–403. https://doi.org/10.1007/s10499-005-9040-z

Jun, J. W., Kim, H. J., Yun, S. K., Chai, J. Y., & Park, S. C. (2014). Eating oysters without risk of vibriosis: Application of a bacteriophage against *Vibrio parahaemolyticus* in oysters. *International Journal of Food Microbiology*, *188*, 31–35. https://doi.org/10.1016/j.ijfoodmicro.2014.07.007

Kang, C. H., Gu, T., & So, J. S. (2018). Possible probiotic lactic acid bacteria isolated from oysters (*Crassostrea gigas*). *Probiotics and Antimicrobial Proteins*, *10*(4), 728–739. https://doi.org/10.1007/s12602-017-9315-5

Karim, M., Zhao, W., Rowley, D., Nelson, D., & Gomez-Chiarri, M. (2013). Probiotic strains for shellfish aquaculture: Protection of eastern oyster, *Crassostrea virginica*, larvae and juveniles against bacterial challenge. *Journal of Shellfish Research*, *32*(2), 401–408. https://doi.org/10.2983/035.032.0220

Katharios, P., Kalatzis, P. G., Kokkari, C., Sarropoulou, E., & Middelboe, M. (2017). Isolation and characterization of a N4-like lytic bacteriophage infecting *Vibrio splendidus*, a pathogen of fish and bivalves. *PLoS ONE*, *12*(12). https://doi.org/10.1371/journal.pone.0190083

Khan, B., Clinton, S. M., Hamp, T. J., Oliver, J. D., & Ringwood, A. H. (2018). Potential impacts of hypoxia and a warming ocean on oyster microbiomes. *Marine Environmental Research*, 139(May), 27–34. https://doi.org/10.1016/j.marenvres.2018.04.018

Kim, H. J., Jun, J. W., Giri, S. S., Chi, C., Yun, S., Kim, S. G., Kim, S. W., Kang, J. W., Han, S. J., Kwon, J., Oh, W. T., & Park, S. C. (2019). Application of the bacteriophage pVco-14 to prevent *Vibrio coralliilyticus* infection in Pacific oyster (*Crassostrea gigas*) larvae. *Journal of Invertebrate Pathology*, *167*. https://doi.org/10.1016/j.jip.2019.107244

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS ONE*, 7(12). https://doi.org/10.1371/journal.pone.0051475

King, W. L., Jenkins, C., Seymour, J. R., & Labbate, M. (a). (2019, January 1). Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment. *Marine Environmental Research*. Elsevier Ltd. https://doi.org/10.1016/j.marenvres.2018.11.007

King, W. L., Siboni, N., Kahlke, T., Green, T. J., Labbate, M., & Seymour, J. R. (b). (2019). A New High Throughput Sequencing Assay for Characterizing the Diversity of Natural *Vibrio* Communities and Its Application to a Pacific Oyster Mortality Event. *Frontiers in Microbiology*, 10, 1–13. https://doi.org/10.3389/fmicb.2019.02907

King, W. L., Jenkins, C., Go, J., Siboni, N., Seymour, J. R., & Labbate, M. (c). (2019). Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. *Microbial Ecology*, 77(2), 502–512. https://doi.org/10.1007/s00248-018-1226-9

King, W. L., Siboni, N., Williams, N. L. R., Kahlke, T., Nguyen, K. V., Jenkins, C., … Labbate, M. (2019). (d). Variability in the composition of pacific oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 µvar disease. *Frontiers in Microbiology*, 10(MAR). https://doi.org/10.3389/fmicb.2019.00473

Kobayashi, M., Msangi, S., Batka, M., Vannuccini, S., Dey, M. M., & Anderson, J. L. (2015). Fish to 2030: *The Role and Opportunity for Aquaculture. Aquaculture Economics and Management*, 19(3), 282–300. https://doi.org/10.1080/13657305.2015.994240

Kong, N., Han, S., Fu, Q., Yu, Z., Wang, L., & Song, L. (2022). Impact of ocean acidification on the intestinal microflora of the Pacific oyster Crassostrea gigas. *Aquaculture*, 546, 737365.

Kozasa, M., Toyocerin Ž (1986). *Bacillus toyoi* as growth promotor for animal feeding. Microbiol. *Aliment. Nutr*. 4, 121–135.

Laroche, O., Symonds, J. E., Smith, K. F., Banks, J. C., Mae, H., Bowman, J. P., & Pochon, X. (2018). Understanding bacterial communities for informed biosecurity and improved larval survival in Pacific oysters. *Aquaculture*, *497*, 164–173. https://doi.org/10.1016/j.aquaculture.2018.07.052

Larsen, A. M., Mohammed, H. H., & Arias, C. R. (2014). Characterization of the gut microbiota of three commercially valuable warmwater fish species. *Journal of Applied Microbiology*, 116(6), 1396–1404. https://doi.org/10.1111/jam.12475

Le, T. H., Truong, T., Tran, L. T., Nguyen, D. H., Pham, T. P. T., & Ng, C. (2023). Antibiotic resistance in the aquatic environments: the need for an interdisciplinary approach. In *International Journal of Environmental Science and Technology* 20(3), 3395–3408. Institute for Ionics. https://doi.org/10.1007/s13762-022-04194-9

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

Li, X., Li, J., Wang, Y., Fu, L., Fu, Y., Li, B., & Jiao, B. (2011). Aquaculture Industry in China: Current State, Challenges, and Outlook. *Reviews in Fisheries Science*, 19(3), 187–200. https://doi.org/10.1080/10641262.2011.573597

Liu, M., Li, Q., Tan, L., Wang, L., Wu, F., Li, L., & Zhang, G. (2023). Host-microbiota interactions play a crucial role in oyster adaptation to rising seawater temperature in summer. *Environmental Research*, 216. https://doi.org/10.1016/j.envres.2022.114585

Liu, M., Li, Q., Xu, W., Wang, L., Wu, F., Tan, L., ... & Zhang, G. (2024). Characterization of water microbiota and their relationship with resident oysters during an oyster mortality event. *Microbiology Spectrum*, 12(10), e02881-23.

Lucas, J, S., Southgate, P, C and Tucker, C, S. (2019). Aquaculture: Farming Aquatic Animals and Plants. John Wiley & Sons Ltd. Third edition.

Lucasson, A., Luo, X., Mortaza, S., de Lorgeril, J., Toulza, E., Petton, B., … Mitta, G. (2020). A core of functionally complementary bacteria colonizes oysters in Pacific Oyster Mortality Syndrome. *BioRxiv*, 2020.11.16.384644. https://doi.org/10.1101/2020.11.16.384644

MacKenzie, M., & Argyropoulos, C. (2023). An Introduction to Nanopore Sequencing: Past, Present, and Future Considerations. In *Micromachines* 14 (2). MDPI. https://doi.org/10.3390/mi14020459

Madhusudana Rao, B., & Lalitha, K. V. (2015). Bacteriophages for aquaculture: Are they beneficial or inimical. In *Aquaculture* 37, 146–154. Elsevier. https://doi.org/10.1016/j.aquaculture.2014.11.039

Maghsodian, Z., Sanati, A. M., Mashifana, T., Sillanpää, M., Feng, S., Nhat, T., & Ramavandi, B. (2022). Occurrence and Distribution of Antibiotics in the Water, Sediment, and Biota of Freshwater and Marine Environments: A Review. In *Antibiotics* 11 (11). MDPI. https://doi.org/10.3390/antibiotics11111461

Martínez Cruz, P., Ibáñez, A. L., Monroy Hermosillo, O. A., & Ramírez Saad, H. C. (2012). Use of Probiotics in Aquaculture. *ISRN Microbiology*, *2012*, 1–13. https://doi.org/10.5402/2012/916845

Martínez-García, M. F., Ruesink, J. L., Grijalva-Chon, J. M., Lodeiros, C., Arreola-Lizárraga, J. A., de la Re-Vega, E., ... & Chávez-Villalba, J. (2022). Socioecological factors related to aquaculture introductions and production of Pacific oysters (*Crassostrea gigas*) worldwide. *Reviews in aquaculture*, 14(2), 613-629.

Mattila, J. M., Zimmer, M., Vesakoski, O., & Jormalainen, V. (2014). Habitat-specific gut microbiota of the marine herbivore *Idotea balthica* (Isopoda). *Journal of Experimental Marine Biology and Ecology*, 455, 22–28. https://doi.org/10.1016/j.jembe.2014.02.010

Md Zoqratt, M. Z. H., Eng, W. W. H., Thai, B. T., Austin, C. M., & Gan, H. M. (2018). Microbiome analysis of Pacific white shrimp gut and rearing water from Malaysia and Vietnam: implications for aquaculture research and management. *PeerJ*, 6, e5826. https://doi.org/10.7717/peerj.5826

Maeda, M., Liao, I.C (1992). Effect of bacterial population on the growth of a prawn larva, Penaeus monodon. *Bull. Natl. Res. Int. Aquacult*. 21, 25 – 29.

McGrath, N., le Roux, J., Whibley, A., Alexander, A., Stewart, R.O., Johnstone, M., Stockin, K.A. and Silander, O.K. (2024). A High-quality Oxford Nanopore Assembly of the Hourglass Dolphin (*Sagmatias cruciger*) *Genome. bioRxiv*, pp.2024-05.

Mignard, S., & Flandrois, J. P. (2006). 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. *Journal of Microbiological Methods*, 67(3), 574–581. https://doi.org/10.1016/j.mimet.2006.05.009

Mondal, H., & Thomas, J. (2022). A review on the recent advances and application of vaccines against fish pathogens in aquaculture. In *Aquaculture International* 30 (4), 1971–2000*. Springer Science and Business Media Deutschland GmbH*. https://doi.org/10.1007/s10499-022-00884-w

Morga, B., Jacquot, M., Pelletier, C., Chevignon, G., Dégremont, L., Biétry, A., … Lamy, J. B. (2021). Genomic Diversity of the Ostreid Herpesvirus Type 1 Across Time and Location and Among Host Species. *Frontiers in Microbiology*, 12(July), 1–13. https://doi.org/10.3389/fmicb.2021.711377

Murchelano, R. A., & Bishop, J. L. (1969). Bacteriological study of laboratory-reared juvenile American oysters (*Crassostrea virginica*). *Journal of Invertebrate Pathology*, 14(3), 321–327. https://doi.org/10.1016/0022-2011(69)90158-X

Mustafa, A., & Piasecki, W. (2005). Factors affecting the susceptibility of sea lice infection in Atlantic salmon. *Annals of Parasitology*, 51(4).

Nachimuthu, R., Madurantakam Royam, M., Manohar, P., & Leptihn, S. (2021). Application of bacteriophages and endolysins in aquaculture as a biocontrol measure. In *Biological Control*, 160. Academic Press Inc. https://doi.org/10.1016/j.biocontrol.2021.104678

Nadarajah, S., & Flaaten, O. (2017). Global aquaculture growth and institutional quality. *Marine Policy*, 84, 142–151. https://doi.org/10.1016/j.marpol.2017.07.018

Nascimento-schulze, J. C., Bean, T. P., Houston, R. D., Sanders, M. B., Lewis, C., & Ellis, R. P. (2021). Optimizing hatchery practices for genetic improvement of marine bivalves, *Reviews in Aquaculture*, 13(4), 2289-2304.

Nicholls, S. M., Quick, J. C., Tang, S., & Loman, N. J. (2019). Ultra-deep, long-read nanopore sequencing of mock microbial community standards. *GigaScience*, 8(5), 1–9. https://doi.org/10.1093/gigascience/giz043

Nielsen, M., Hansen, B. W., & Vismann, B. (2017). Feeding traits of the European flat oyster, *Ostrea edulis*, and the invasive Pacific oyster, *Crassostrea gigas*. *Marine Biology*, 164(1), 1–10. https://doi.org/10.1007/s00227-016-3041-5

Ninawe, A. S., Sivasankari, S., Ramasamy, P., Kiran, G. S., & Selvin, J. (2020). Bacteriophages for aquaculture disease control. In *Aquaculture International*, 28, (5), 1925–1938 https://doi.org/10.1007/s10499-020-00567-4

Noman, M., Kazmi, S. S. U. H., Saqib, H. S. A., Fiaz, U., Pastorino, P., Barcelò, D., Tayyab, M., Liu, W., Wang, Z., & Yaseen, Z. M. (2024). Harnessing probiotics and prebiotics as eco-friendly solution for cleaner shrimp aquaculture production: A state of the art scientific consensus. In *Science of the Total Environment*, 915. Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2024.169921

Ogier, J. C., Pagès, S., Galan, M., Barret, M., & Gaudriault, S. (2019). RpoB, a promising marker for analyzing the diversity of bacterial communities by amplicon sequencing. *BioRxiv*, 1–16. https://doi.org/10.1101/626119

Oglend, A. (2020). Challenges and opportunities with aquaculture growth. *Aquaculture Economics and Management*, 24(2), 123–127. https://doi.org/10.1080/13657305.2019.1704937

Okeke, E. S., Chukwudozie, K. I., Nyaruaba, R., Ita, R. E., Oladipo, A., Ejeromedoghene, O., Atakpa, E. O., Agu, C. V., & Okoye, C. O. (2022). Antibiotic resistance in aquaculture and aquatic organisms: a review of current nanotechnology applications for sustainable management. In *Environmental Science and Pollution Research*, 29 (46), 69241–69274. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s11356-022-22319-y

Okocha, R. C., Olatoye, I. O., & Adedeji, O. B. (2018). Food safety impacts of antimicrobial use and their residues in aquaculture. In *Public Health Reviews*, 39 , 1-22. BioMed Central Ltd. https://doi.org/10.1186/s40985-018-0099-2

Oliveira, J., Castilho, F., Cunha, A., & Pereira, M. J. (2012). Bacteriophage therapy as a bacterial control strategy in aquaculture. In *Aquaculture International*, 20 (5), 879–910. https://doi.org/10.1007/s10499-012-9515-7

Ossai, S., Ramachandran, P., Ottesen, A., & Reed, E. (2017). Microbiomes of American Oysters Two Sites in the Chesapeake Bay. *Genome Anno*, 5(30), 1–2.

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in Aquaculture*, 11(4), 1398–1417. https://doi.org/10.1111/raq.12299

Paillard, C., Gueguen, Y., Wegner, K. M., Bass, D., Pallavicini, A., Vezzulli, L., & Arzul, I. (2022). Recent advances in bivalve-microbiota interactions for disease prevention in aquaculture. In *Current Opinion in Biotechnology*, 73, pp. 225–232. Elsevier Ltd. https://doi.org/10.1016/j.copbio.2021.07.026

Parker, R.B. (1974). Probiotics, the Other Half of Antibiotic Story. *Animal Nutrition & Health*, 29, 4-8.

Paul-Pont, I., Dhand, N. K., & Whittington, R. J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. *Aquaculture*, 412, 202–214. https://doi.org/10.1016/j.aquaculture.2013.07.038

Pauly, D., & Zeller, D. (2017). Comments on FAOs State of World Fisheries and Aquaculture (SOFIA 2016). *Marine Policy*, 77, 176–181. https://doi.org/10.1016/j.marpol.2017.01.006

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., ... & Keck, N. (2012). Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. *Aquaculture Environment Interactions*, 2(3), 215-237.

Pernet, F., Lupo, C., Bacher, C., & Whittington, R. J. (2016). Infectious diseases in oyster aquaculture require a new integrated approach. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 371(1689). https://doi.org/10.1098/rstb.2015.0213

Pettersen, A. K., Marshall, D. J., & White, C. R. (2018). Understanding variation in metabolic rate. In *Journal of Experimental Biology*, 21 (1). Company of Biologists Ltd. https://doi.org/10.1242/jeb.166876

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., & Le Roux, F. (2015). *Crassostrea gigas* mortality in France: The usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Frontiers in Microbiology*, 6(JUL), 1–10. https://doi.org/10.3389/fmicb.2015.00686

Petton, B., Destoumieux-Garzón, D., Pernet, F., Toulza, E., de Lorgeril, J., Degremont, L., & Mitta, G. (2021). The Pacific Oyster Mortality Syndrome, a Polymicrobial and Multifactorial Disease: State of Knowledge and Future Directions. *Frontiers in Immunology*, 12, 1–10. https://doi.org/10.3389/fimmu.2021.630343

Pierce, M. L., & Evan, J. (2019). Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) and the Blue Mussel (*Mytilus edulis*): Temporal Variation and the Influence of Marine Aggregate-Associated Microbial Communities. *American Society for Microbiology*, *mSphere*, 4(6), 1–17.

Pimentel, Z. T., Dufault-Thompson, K., Russo, K. T., Scro, A. K., Smolowitz, R. M., Gomez-Chiarri, M., & Zhang, Y. (2021). Microbiome Analysis Reveals Diversity and Function of

Mollicutes Associated with the Eastern Oyster, *Crassostrea virginica. MSphere*, *6*(3). https://doi.org/10.1128/msphere.00227-21

Plough, L. V. (2018). Fine-scale temporal analysis of genotype-dependent mortality at settlement in the Pacific oyster *Crassostrea gigas. Journal of Experimental Marine Biology and Ecology*, 501, 90–98. https://doi.org/10.1016/j.jembe.2018.01.006

Prado S, Romalde JL, Montes J, Barja JL (2005). Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. *Dis Aquat Organ*, 67(3), 209-215. https://doi: 10.3354/dao067209. PMID: 16408836.

Proctor, L. M. (2011). The human microbiome project in 2011 and beyond. *Cell Host and Microbe*, 10(4), 287–291. https://doi.org/10.1016/j.chom.2011.10.001

Rang, F. J., Kloosterman, W. P., & de Ridder, J. (2018). From squiggle to basepair: Computational approaches for improving nanopore sequencing read accuracy. *Genome Biology*, 19(1), 1–11. https://doi.org/10.1186/s13059-018-1462-9

Rasheeda, M. K., Rangamaran, V. R., Srinivasan, S., Ramaiah, S. K., Gunasekaran, R., Jaypal, S., … Ramalingam, K. (2017). Comparative profiling of microbial community of three economically important fishes reared in sea cages under tropical offshore environment. *Marine Genomics*, 34, 57–65. https://doi.org/10.1016/j.margen.2017.04.003

Ray, N. E., Maguire, T. J., Al-Haj, A. N., Henning, M. C., & Fulweiler, R. W. (2019). Low greenhouse gas emissions from oyster aquaculture. *Environmental Science and Technology*, 53(15), 9118–9127. https://doi.org/10.1021/acs.est.9b02965

Richard, M., Rolland, J. L., Gueguen, Y., de Lorgeril, J., Pouzadoux, J., Mostajir, B., … Roque d'Orbcastel, E. (2021). In situ characterisation of pathogen dynamics during a Pacific oyster mortality syndrome episode. *Marine Environmental Research*, 165. https://doi.org/10.1016/j.marenvres.2020.105251

Richards, G. P. (2014). Bacteriophage remediation of bacterial pathogens in aquaculture: a review of the technology. *Bacteriophage*, *4*(4), e975540. https://doi.org/10.4161/21597081.2014.975540

Richards, G. P., Watson, M. A., Needleman, D. S., Church, K. M., & Häse, C. C. (2015). Mortalities of Eastern And Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Applied and Environmental Microbiology*, 81(1), 292–297. https://doi.org/10.1128/AEM.02930-14

Rico-Villa, B., Pouvreau, S., & Robert, R. (2009). Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae, *Crassostrea gigas. Aquaculture*, 287(3-4), 395-401.

Ringø, E. (2020). Probiotics in shellfish aquaculture. In *Aquaculture and Fisheries*, 5 (1), 1–27). *KeAi Communications Co*. https://doi.org/10.1016/j.aaf.2019.12.001

Ringø, E., Olsen, R. E., Gifstad, T., Dalmo, R. A., Amlund, H., Hemre, G. I., & Bakke, A. M. (2010). Prebiotics in aquaculture: A review. In *Aquaculture Nutrition* 6 (2), 117–136 https://doi.org/10.1111/j.1365-2095.2009.00731.x

Risely, A. (2020). Applying the core microbiome to understand host–microbe systems. *Journal of animal ecology*, 89(7), 1549-1558.

Roch, P. (1999). Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture*, 172(1–2), 125–145. https://doi.org/10.1016/S0044-8486(98)00439-6

Sainz-Hernández, J. C., & Maeda-Martínez, A. N. (2005). Sources of *Vibrio* bacteria in mollusc hatcheries and control methods: A case study. *Aquaculture Research*, *36*(16), 1611–1618. https://doi.org/10.1111/j.1365-2109.2005.01386.x

Salgueiro, V., Reis, L., Ferreira, E., Botelho, M. J., Manageiro, V., & Caniça, M. (2021). Assessing the bacterial community composition of bivalve mollusks collected in aquaculture farms and respective susceptibility to antibiotics. *Antibiotics*, *10*(9). https://doi.org/10.3390/antibiotics10091135

Sánchez-Ortiz, A. C., Mazón-Suástegui, J. M., del C. Flores-Miranda, M., Luna-González, A., Ochoa, N., Melgar-Valdés, C. E., & Campa-Córdova, Á. I. (2020). Probiotic Bacterium and Microalga Interaction on Rearing Kumamoto Oyster *Crassostrea sikamea* Spat. *Current Microbiology*, *77*(10), 2758–2765. https://doi.org/10.1007/s00284-020-02076-2

Scanes, E., Parker, L. M., Seymour, J. R., Siboni, N., Dove, M. C., O'Connor, W. A., & Ross, P. M. (a). (2021). Microbiomes of an oyster are shaped by metabolism and environment. *Scientific Reports*, 11(1), 1–7. https://doi.org/10.1038/s41598-021-00590-2

Scanes, E., Parker, L. M., Seymour, J. R., Siboni, N., King, W. L., Wegner, K. M., Dove, M. C., O'Connor, W. A., & Ross, P. M. (b). (2021). Microbiome response differs among selected lines of Sydney rock oysters to ocean warming and acidification. *FEMS Microbiology Ecology*, *97*(8), 1–13. https://doi.org/10.1093/femsec/fiab099

Scanes, E., Parker, L. M., Seymour, J. R., Siboni, N., King, W. L., Danckert, N. P., … Ross, P. M. 2021. (c). Climate change alters the haemolymph microbiome of oysters. *Marine Pollution Bulletin*. 164, 111991. https://doi.org/10.1016/j.marpolbul.2021.111991

Scanes, E., Ross, P. M., Seymour, J. R., Siboni, N., Dove, M. C., O'Connor, W. A., Dittes, C., & Parker, L. M. (2023). Transgenerational transfer of the microbiome is altered by ocean acidification in oyster larvae. *Aquaculture*, 565. https://doi.org/10.1016/j.aquaculture.2022.739153

Schar, D., Klein, E. Y., Laxminarayan, R., Gilbert, M., & Van Boeckel, T. P. (2020). Global trends in antimicrobial use in aquaculture. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-78849-3

Schrobback, P., Pascoe, S., & Coglan, L. (2018). Quantifying the economic impact of climate change and market dynamics: The case of Australia's sydney rock oyster industry. *Marine Resource Economics*, 33(2), 155–175. https://doi.org/10.1086/697419

Schulz, P., Pajdak-Czaus, J., & Siwicki, A. K. (2022). In Vivo Bacteriophages' Application for the Prevention and Therapy of Aquaculture Animals—Chosen Aspects. In *Animals* 12 (10). MDPI. https://doi.org/10.3390/ani12101233

Schulze, A. D., Alabi, A. O., Tattersall-Sheldrake, A. R., & Miller, K. M. (2006). Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture*, 256(1–4), 50–73. https://doi.org/10.1016/j.aquaculture.2006.02.008

Sharifuzzaman, S. M., & Austin, B. (2017). Probiotics for disease control in aquaculture. In *Diagnosis and Control of Diseases of Fish and Shellfish*, 189–222. Wiley. https://doi.org/10.1002/9781119152125.ch8

Sieiro, C., Areal-hermida, L., Pichardo-gallardo, Á., Almuiña-González, R., de Miguel, T., Sánchez, S., Sánchez-pérez, Á., & Villa, T. G. (2020). A hundred years of bacteriophages: Can phages replace antibiotics in agriculture and aquaculture? In *Antibiotics*, 9 (8), 1–30. MDPI AG. https://doi.org/10.3390/antibiotics9080493

Smárason, B. Ö., Alriksson, B., & Jóhannsson, R. (2019). Safe and sustainable protein sources from the forest industry – The case of fish feed. *Trends in Food Science and Technology*, 84, 12–14. https://doi.org/10.1016/j.tifs.2018.03.005

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. In *Expert Review of Vaccines*, 4, (1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Stechele, B., Maar, M., Wijsman, J., Van der Zande, D., Degraer, S., Bossier, P., & Nevejan, N. (2022). Comparing life history traits and tolerance to changing environments of two oyster species (*Ostrea edulis* and *Crassostrea gigas*) through Dynamic Energy Budget theory. *Conservation Physiology*, 10(1), coac034.

Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., … Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology*, 110(2), 141–157. https://doi.org/10.1016/j.jip.2012.03.013

Stevick, R. J., Post, A. F., & Gómez-Chiarri, M. (2021). Functional plasticity in oyster gut microbiomes along a eutrophication gradient in an urbanized estuary. *Animal Microbiome*, 3(1). https://doi.org/10.1186/s42523-020-00066-0

Sun, R., Chen, J., Pan, C., Sun, Y., Mai, B., & Li, Q. X. (2020). Antibiotics and food safety in aquaculture. *Journal of Agricultural and Food Chemistry*, *68*(43), 11908–11919. https://doi.org/10.1021/acs.jafc.0c03996

Tacon, A. G. J. (2020). Trends in Global Aquaculture and Aquafeed Production: 2000–2017. *Reviews in Fisheries Science and Aquaculture*, 28(1), 43–56. https://doi.org/10.1080/23308249.2019.1649634

Tidwell, J. H., & Allan, G. L. (2001). Fish as food: Aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries. *EMBO Reports*, 2(11), 958–963. https://doi.org/10.1093/embo-reports/kve236

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O. T., Nilsen, F., … Jackson, D. (2013). Salmon lice - impact on wild salmonids and salmon aquaculture. J*ournal of Fish Diseases*, 36(3), 171–194. https://doi.org/10.1111/jfd.12061

Tout, J., Jeffries, T. C., Webster, N. S., Stocker, R., Ralph, P. J., & Seymour, J. R. (2014). Variability in microbial community composition and function between different niches within a coral reef. *Microbial Ecology*, 67(3), 540–552. https://doi.org/10.1007/s00248-013-0362-5

Trabal Fernández, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Ascencio-Valle, F., & Romero, J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiology Ecology*, 88(1), 69–83. https://doi.org/10.1111/1574-6941.12270

Tsai, H. Y., Matika, O., Edwards, S. M. K., Antolín-Sánchez, R., Hamilton, A., Guy, D. R., Tinch, A. E., Gharbi, K., Stear, M. J., Taggart, J. B., Bron, J. E., Hickey, J. M., & Houston, R. D. (2017). Genotype imputation to improve the cost-efficiency of genomic selection in farmed Atlantic salmon. *G3: Genes, Genomes, Genetics*, *7*(4), 1377–1383. https://doi.org/10.1534/g3.117.040717

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The Human Microbiome Project. Nature, 449(7164), 804–810. https://doi.org/10.1038/nature06244

Unzueta-Martínez, A. (2022). Determining the Composition of Resident and Transient Members of the Oyster Microbiome. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.828692

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, 4(1). https://doi.org/10.1186/s42523-022-00186-9

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., & Knight, R. (2012). Defining the human microbiome. *Nutrition Reviews*, 70(SUPPL. 1). https://doi.org/10.1111/j.1753-4887.2012.00493.x

Ushijima, B., Richards, G. P., Watson, M. A., Schubiger, C. B., & Häse, C. C. (2018). Factors affecting infection of corals and larval oysters by *Vibrio coralliilyticus*. PLoS ONE, *13*(6). https://doi.org/10.1371/journal.pone.0199475

Vignier, J., Laroche, O., Rolton, A., Wadsworth, P., Kumanan, K., Trochel, B., Pochon, X., & King, N. (2021). Dietary Exposure of Pacific Oyster (*Crassostrea gigas*) Larvae to Compromised Microalgae Results in Impaired Fitness and Microbiome Shift. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.706214

Vogeler, S., Bean, T. P., Lyons, B. P., & Galloway, T. S. (2016). Dynamics of nuclear receptor gene expression during Pacific oyster development. *BMC Developmental Biology*, 16(1), 1–13. https://doi.org/10.1186/s12861-016-0129-6

Vogeler, S., Carboni, S., Li, X., Nevejan, N., Monaghan, S. J., Ireland, J. H., & Joyce, A. (2020). Bivalves are NO different: nitric oxide as negative regulator of metamorphosis in the Pacific oyster, *Crassostrea gigas*. *BMC Developmental Biology*, 20(1), 1–18. https://doi.org/10.1186/s12861-020-00232-2

Wallace, R. K., Waters, P., & Rikard, F. S. (2008). Oyster hatchery techniques.

Wang, D., Loor, A., De Bels, L., Van Stappen, G., Van den Broeck, W., & Nevejan, N. (2021). Dynamic immune response to vibriosis in pacific oyster *Crassostrea gigas* larvae during the infection process as supported by accurate positioning of GFP-tagged vibrio strains. *Microorganisms*, 9(7). https://doi.org/10.3390/microorganisms9071523

Wee, W., Abdul Hamid, N. K., Mat, K., Khalif, R. I. A. R., Rusli, N. D., Rahman, M. M., Kabir, M. A., & Wei, L. S. (2024). The effects of mixed prebiotics in aquaculture: A review. In *Aquaculture and Fisheries* 9 (1), 28–34. KeAi Communications Co. https://doi.org/10.1016/j.aaf.2022.02.005

Wegner, K. M., Volkenborn, N., Peter, H., & Eiler, A. (2013). Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiology*, *13*(1). https://doi.org/10.1186/1471-2180-13-252

Wendling, C. C., Batista, F. M., & Wegner, K. M. (2014). Persistence, seasonal dynamics and pathogenic potential of *vibrio* communities from pacific oyster hemolymph. *PLoS ONE*, 9(4). https://doi.org/10.1371/journal.pone.0094256

Wendling, C. C., Fabritzek, A. G., & Wegner, K. M. (2017). Population-specific genotype x genotype x environment interactions in bacterial disease of early life stages of Pacific oyster larvae. *Evolutionary Applications*, *10*(4), 338–347. https://doi.org/10.1111/eva.12452

Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Tse, H., & Yuen, K. Y. (2008). Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, 14(10), 908–934. https://doi.org/10.1111/j.1469-0691.2008.02070.x

Yang, L., Yang, Q., Hu, R. G., Cong, W., Li, S., & Kang, Y. H. (2024). The evaluation of bacteriophage therapy in aquaculture: A systematic review and meta-analysis. In *Aquaculture*, 588. Elsevier B.V. https://doi.org/10.1016/j.aquaculture.2024.740925

Yeh, H., Skubel, S. A., Patel, H., Cai Shi, D., Bushek, D., & Chikindas, M. L. (2020). From Farm to Fingers: an Exploration of Probiotics for Oysters, from Production to Human

Consumption. *Probiotics and Antimicrobial Proteins*, 12(2), 351–364. https://doi.org/10.1007/s12602-019-09629-3

You, W., & Hedgecock, D. (2019). Boom-and-bust production cycles in animal seafood aquaculture. *Reviews in Aquaculture*, 11(4), 1045–1060. https://doi.org/10.1111/raq.1227

Zidane, N., Rodrigues, C., Bouchez, V., Rethoret-Pasty, M., Passet, V., Brisse, S. and Crestani, C., 2024. Accurate genotyping of three major respiratory bacterial pathogens with ONT R10. 4.1 long-read sequencing. *bioRxiv*, 2024-10.

Zhong, K. X., Chan, A. M., Collicutt, B., Daspe, M., Finke, J. F., Foss, M., Green, T. J., Harley, C. D. G., Hesketh, A. V., Miller, K. M., Otto, S. P., Rolheiser, K., Saunders, R., Sutherland, B. J. G., & Suttle, C. A. (2024). The prokaryotic and eukaryotic microbiome of Pacific oyster spat is shaped by ocean warming but not acidification. *Applied and Environmental Microbiology*, *90* (4). https://doi.org/10.1128/aem.00052-24

Zhu, Y. G., Zhu, D., Rillig, M. C., Yang, Y., Chu, H., Chen, Q. L., Penuelas, J., Cui, H. L., & Gillings, M. (2023). *Ecosystem Microbiome Science*. *MLife*, *2*(1), 2–10. https://doi.org/10.1002/mlf2.12054

Chapter two: First investigation of the environmental microbiome throughout a wild bivalve mollusc spawning event



Photograph taken by Kallen Sullivan

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First investigation of the environmental microbiome throughout a wild bivalve mollusc spawning event

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Abstract:

The microbiome plays a key role in animal health, and is an important aspect of any natural or farmed ecosystem. Here we present an environmental microbiome study of *Ostrea edulis*, during a natural wild spawning event. The oyster larval microbiome has been previously reported to be distinct to that of the external environment. It was therefore hypothesised that larval abundance would be correlated with specific microbial signatures. Water samples were collected in volumes of 200 L throughout a natural spawning event of *O. edulis* at Loch Ryan, Scotland, UK. Each water sample was condensed on a 90 µm mesh (retaining larvae, phytoplankton and other organic matter). This condensed sample was used for further processing. Samples were collected on 4 different dates from June to September 2019, across 8 different sampling sites on the Loch at mid, bottom and surface levels within the water column to remove effects of salinity and tidal fluctuations. Larval count data was obtained from these samples before full-length sequencing of the 16S rRNA gene using Oxford Nanopore Technologies. Significant microbial differences were only found between samples collected on different dates, and not at different sites or water column depths. Differences in the microbiome throughout the spawning season were driven by changes in the abundance

of certain taxa, most notably those belonging to the *Rhodobacteraceae* family. Inverse abundance profiles of *Rhodobacteraceae* and *Vibrio* over time are also discussed. This study provides important microbial baseline data about the spawning environment of *O. edulis*

Introduction:

The European flat oyster (*Ostrea edulis*) has a wide natural distribution (Liu et al, 2011) inhabiting shallow estuaries along the coastlines of Europe, through to the Mediterranean and as far as the Black Sea (Farinas-Franco et al, 2018). Natural populations of *O. edulis* were once plentiful in open sea environments around the UK before they were overfished in the 1800's (Thurstan *et al*. 2013). Remnant populations have, subsequently, been in fast decline since the late 19th century (Kennedy and Roberts, 1999; Laing, Walker and Areal, 2006). This has largely been due to anthropogenic activities such as overexploitation alongside the emergence of parasitic diseases (Engelsma et al, 2010; Pouvreau et al, 2023; Smyth et al, 2016) such as *Bonamia ostreae* and *Martelia refringens*. These factors have resulted in *O. edulis* becoming a threatened species, as defined by Oslo and Paris Conventions (OSPAR) (Guy, Smyth and Roberts, 2019). Sizeable UK populations of *O. edulis* in the UK now only occur in the Essex Estuaries, Solent, River Fal (England), Strangford Lough (Northern Ireland) and Loch Ryan (Scotland) (Allison et al, 2019 and Thurstan et al, 2013).

Ostrea edulis can provide a sustainable protein source, from both fisheries and aquaculture production, which was estimated at 3,305 tonnes per year in Europe between 2006-2015 (Segvic-Bubic et al, 2020). Natural oyster beds also offer a range of ecosystem services including substrate stabilisation, water filtration and habitat formation for various species (Boutet et al, 2022; Thomas et al, 2022; Kennon et al 2023). Due to the wide range of ecological benefits provided by *O. edulis*, there are now many restoration efforts in place which use translocation to restore natural populations (Colsoul et al, 2021). However, the production of *O. edulis* for either aquaculture or restoration purposes is heavily reliant upon the continuous production of good quality seedstock in the form of spat (Colsoul et al, 2021). This production is often hindered by large scale mortality events occurring just after spawning (within the larval stages of the life cycle). Some of these mortality events can be linked to specific disease-causing pathogens, however many go unrecorded with no known causative

agent. This is largely due to the multifaceted nature of mortality events with a range of environmental parameters, pathogens and host microbiomes interacting together to influence disease status and in turn host health. This not only makes causes of mortality events difficult to record, but also challenging to prevent.

Unlike the more widely farmed Pacific oyster (*Crassostrea or Magallana gigas)*, *O. edulis* is larviparous with fertilisation, embryonic and early larval development happening within the mantle cavity of the female (Joyce et al, 2013). With the majority of mortality events happening during larval stages, research on spawning events is especially important. An under researched area within this is the microbiome of *O. edulis* during a spawning event. In the case of *C. gigas*, it is already reported that the species has a core microbiome with distinct species residing in different tissues (King et al, 2012). However, very little work has been done to investigate the microbiome of *O. edulis*, or its surrounding environments, at any life stage. Such research, especially during larval stages, could help provide useful information about successful natural and commercial *O. edulis* spawning events. This in turn would be key to the success of seed production, restoration goals and aquaculture of the species.

Loch Ryan Oyster Fishery Co. Ltd (LROFC) is the last remaining native oyster fishery in Scotland, UK, operating since 1701 (Chapman et al, 2021). This fishery offers the opportunity to investigate the environmental microbiome associated with a natural Scottish spawning season. The natural spawning season of *O. edulis* in Loch Ryan occurs between June and September, as triggered by increasing water temperatures (Joyce et al, 2013). Indeed, Chapman et al, 2021 concluded temperature sum (sum total of degrees per day above 7˚C) as the most significant predictor of larval abundance. As well as increasing water temperatures, factors such as lunar cycle have also been reported to influence larval abundance (Chapman et al, 2021). There is however no current evidence to suggest that certain microbial signatures of the water microbiome could be a predictor of larval abundance during a spawning season.

Female oysters may produce up to one million eggs which are fertilised within the mantle cavity by indrawn sperm from nearly male oysters (Hayer et al, 2019). Following initial fertilisation, larvae remain within the mantle cavity for 8 – 10 days before being released into the water column. Larvae then spend another 8 – 10 days free-swimming within the water column before settling on substrate (Hayer et al, 2019). Under favourable conditions (water

temperature and food availability), male *O. edulis* individuals are change sex mid-spawning season (Cole, 1941). It is therefore possible for the same oyster to spawn more than once per spawning season (Cole, 1941).

We aimed to report upon the microbiome of a natural *O. edulis* spawning event through analysing bacterial identification and abundance data from a range of samples collected from different dates, locations and depths within the water column. The larval microbiome of oyster species such as *Crassostrea virginica* have been previously reported to be distinct to that of their surrounding water (Arfken et al, 2021). It was therefore hypothesised that samples with a higher larval abundance would drive a strong microbial signature associated with the larvae. This research builds upon previous analysis of the same spawning event conducted by Chapman et al, (2021) where temporal and spatial abundance patterns of *O. edulis* larvae were analysed across the loch.

Methods:

Sample collection:

Weekly water samples were collected from Loch Ryan between the 7th June to 24th September 2019 as described by Chapman et al, (2021). This sampling time frame was chosen to cover the native oyster spawning season as comprehensively as possible. Water samples were collected across 8 different sites in the Loch, covering 4 sites from known oyster fishing grounds off Lefnoll Point, and 4 sites from non-oyster bed habitats (see Figure 1a, Chapman et al, 2021). The location of all sample sites visited on Loch Ryan in relation to the oyster beds can be seen in Figure 1. To remove effects of salinity and tidal fluctuations, water was collected from the surface, mid and bottom points within the water column using the pump and trap method (see Figure 2. Chapman et al, 2021). Each sample filtered 200 L of water through a 90 µm mesh. This resulted in a condensed sample with a maximum volume of 50 mL which contained larvae (of *O. edulis* and other bivalve species), plankton and ultimately any other organic material retained by the mesh. This condensed sample was used for all further processing described. Each samples was used to conduct larval counts of both *O.*

edulis and other bivalves such as cockles, mussels and saddle oysters (Chapman et al, 2021). Water samples were then frozen in a -20°C freezer.

To aid in the downstream analysis, count data of total bivalves and *O. edulis* larvae were used to assign each individual water sample a “low”, “medium” or “high” count category for these two factors. This was done by dividing the highest count number recorded for bivalves and *O. edulis* by 3 in order to set threshold values for each category. The majority of count data for both bivalves and *O. edulis* were categorised at low, with just 4 and 2 samples belonging in the high categories for bivalve and *O. edulis* counts respectively.

A range of physicochemical parameters were recorded at the time of sample collection. Loggers measured water temperature, conductivity, and dissolved oxygen. National Oceanic and Atmospheric Administration (NOAA) daily sea surface temperature allowed for the calculation of ‘temperature sum’ as defined in Chapman et al, 2021. The tidal range and lunar cycle were also recorded for each of the sampling dates.

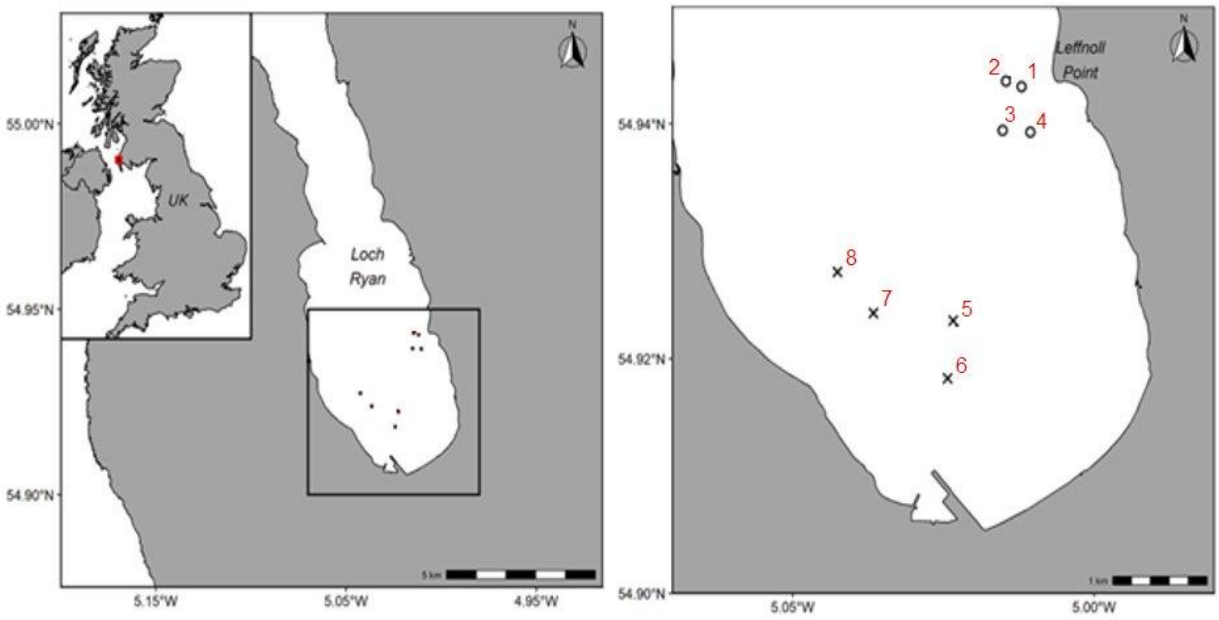


Figure 1: Position of Loch Ryan within the UK, and a map of the loch indicating the location of sampling sites used. Sites marked with an ‘X’ indicate locations off the oyster bed and ‘O’ on the oyster bed.

Molecular biology:

After the initial filtration and freezing, thawed water samples were thoroughly mixed by vortexing for 20 seconds. 15 ml of each was added to a 50 ml falcon tube containing 33 ml of

absolute ethanol and 1.5 ml of 3 M sodium acetate. After 1 hour incubation at -80°C, tubes were centrifuged at 4°C, 4000 rpm for 30 minutes to pellet any cellular material and free DNA. Supernatant was poured off and ethanol allowed to evaporate for 15 mins before 2 ml Liftons buffer with 100 µg/ml Proteinase K was added. This solution was lysed at 55°C for 1 hour, then spun down. 400 µl of supernatant was mixed with 800 µl of Qiagen AL buffer with ethanol, loaded onto a Qiagen spin column and cleaned by following the remainder of the Qiagen blood and tissue kit tissue protocol (Qiagen, Hilden, Germany). In a 96 well plate set up, each DNA sample was then quantified using the QuantiFluor dsDNA system from Promega (Promega, Madison, US) before using as template for amplification of full-length 16S rRNA gene by PCR. This PCR was optimised by using universal 16S primers at a working concentration of 10 µM adapted from Callahan et al, 2019 to also detect bacteria of the *Vibrio* genus: F= AGRGTTYGATYMTGGCTCAG R= VGYTACCTTGTTACGACTT with Phusion High-Fidelity DNA polymerase from APExBIO (ApexBIO, Texas, US). Three positive controls were included by the use of *Streptococcus* bacterial DNA, alongside four negative controls of nuclease free water. The PCR reaction was run with an initial denaturation step at 98°C for 30 seconds, followed by 34 cycles of 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 50 seconds in a PTC-225 Peltier MJ Research Thermocycler (Marshall Scientific, Hampton, US). A total PCR reaction volume was 20 µl was made, incorporating 12.8 µl of template DNA. The subsequent PCR product was then visualised via gel electrophoresis to check for DNA fragments of 1466 base pairs before purifying with the High Prep PCR clean up system (MagBio, Maryland, US). Each DNA sample was then re-quantified using Qubit dsDNA BR Assay kit (Invitrogen, Massachusetts, US) and standardised to ~200 fmol through dilution with nuclease free water. The SQK-LSK110 sequencing ligation kit and EXP-NBD196 Native Barcode Expansion kit (Oxford Nanopore Technologies, Oxford, UK), were then used to perform library preparation with metabarcoding on each of the samples. The pooled library was then loaded across 4 separate ONT MinION R10 flow cells for 48 hours, obtaining a total of 98.681 GB of data.

Data handling and analysis:

High accuracy basecalling was performed using Guppy v.6.1.7 (ONT) and reads were quality controlled by inspecting the average quality and length with Nanoplot version 1.4.0 (De coster et al, 2018). Trimming of primers from reads was then preformed using Cutadapt v.3.4

(Martin, 2011), with a maximum error rate of 0.15 and minimum overlap of 20 base pairs (bp). Reads shorter than 1,300 bp and longer than 1,600 bp were also discarded. Porechop v0.2.4 (Wick et al, 2017) was then used to trim reads of chimeras and adapter sequences. Microbial taxonomy was then identified using Kraken2 v2.1.1 (Wood and Salzberg, 2014), where 2,112,739 reads were aligned against the SILVA138 database (Wood, Lu and Langmead, 2019). Using memory mapping, reads were aligned by breaking sequences into 16-kmers for identification, with matches evaluated at a confidence score of 0.01.

Downstream data analysis was carried out in R studio v4.2.0 (RStudio Team, 2022) using the Phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2014) packages. This included calculation of observed and Choa1 alpha diversity measures which were then tested for significant differences with a Kruskal-Wallis test. The observed alpha diversity measures were used to report the species richness of a sample, defined as the number of different OTUs represented in the microbial community. The Chao1 alpha diversity measure is used as an estimator of total species richness (not just the observed number). This metric corrects for the number of unseen species based on the number of singletons observed within a sample. The Kruskal-Wallis test performed compared the average Chao1 values for samples collected across each of the four dates of sample collection.

Control and outlying samples were identified from non-metric multidimensional scaling (NMDS) analysis and excluded, leaving 85 samples in the results presented. All NMDS analysis was conducted using Bray-Curtis dissimilarity measures. Use of the Vegan package (Oksanen, 2012) in R Studio also allowed for a PERMANOVA statistical tests to be performed. This allowed for statistical significances of the differences between the microbial compositions of samples collected at different site, depths, dates, or larval count categories to be calculated. A Weighted phylogenetic tress (see Figure 3), was created using the Pavian web application tool (Breitwieser and Salzberg, 2020).

Results:

Total bivalve larval counts (general bivalves) varied between samples from 0 to 2,997 and for *O. edulis*, between 0 to 1,015 (Figure 2). The two samples which gave the highest counts of *O. edulis* and general bivalve larvae were both taken from site 8 on 11/07/2019. The mean number of general bivalve and *O. edulis* larvae found across each of these 8 sites on the 4

dates analysed is also shown in Figure 2. Overall, *O. edulis* abundance was lowest on the later dates but varied significantly between each site.

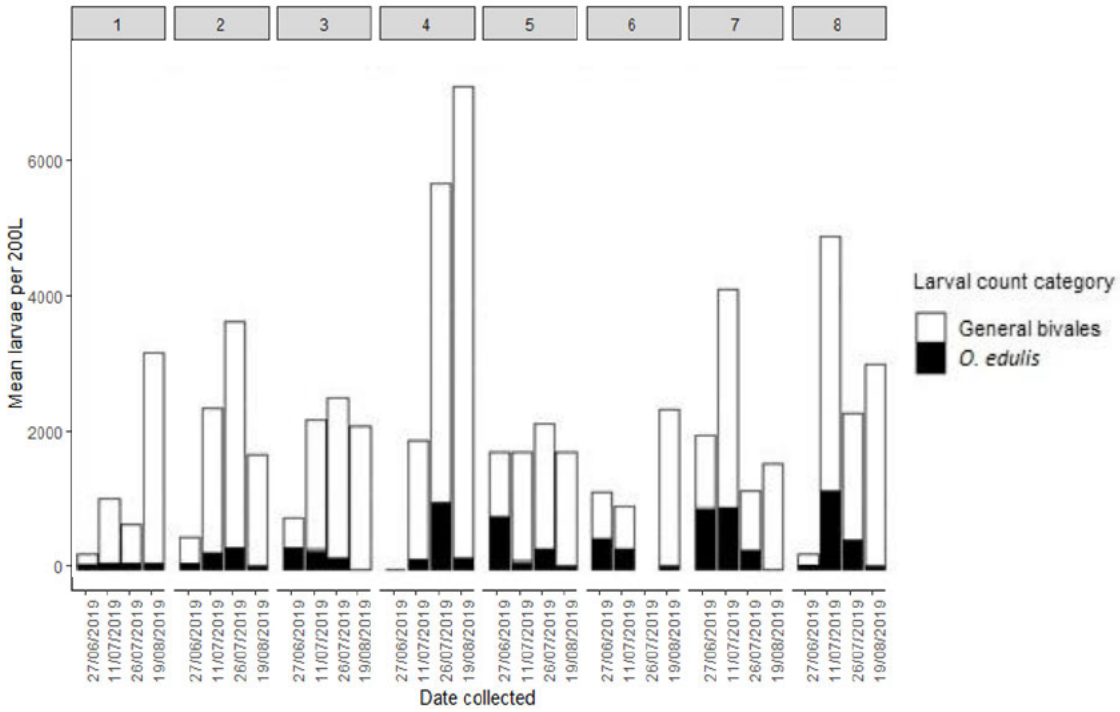


Figure 2: Mean larval count data from water samples collected on 27/06/2019, 11/07/2019, 26/07/2019 and 19/08/2019 at each of the 8 sampling sites. Bars in white indicate general bivalve counts including *O. edulis* as well as cockle, mussel and saddle oysters. Bars in black indicate counts of *O. edulis* only.

The bacterial taxonomic composition found throughout the complete dataset is shown in Figure 3. At the family level, Rhodobacteraceae had the most matched reads followed by Flavobacteriaceae. The top 15 most abundant operational taxonomic units (OTUs) amongst the sample set were identified as belonging to the Halomondaceae, Rhodobacteraceae, Flavobacteriaceae, Cryomorphaceae, Vibrionaceae, Arcobacteraceae, Shewanellaceae, Moraxellaceae or Pseudoalteromonadaceae families (see Figure 4). Figure 4 shows some samples clustered together according to relative abundance of OTUs within the Rhodobacteraceae and Arcobacteraceae families.

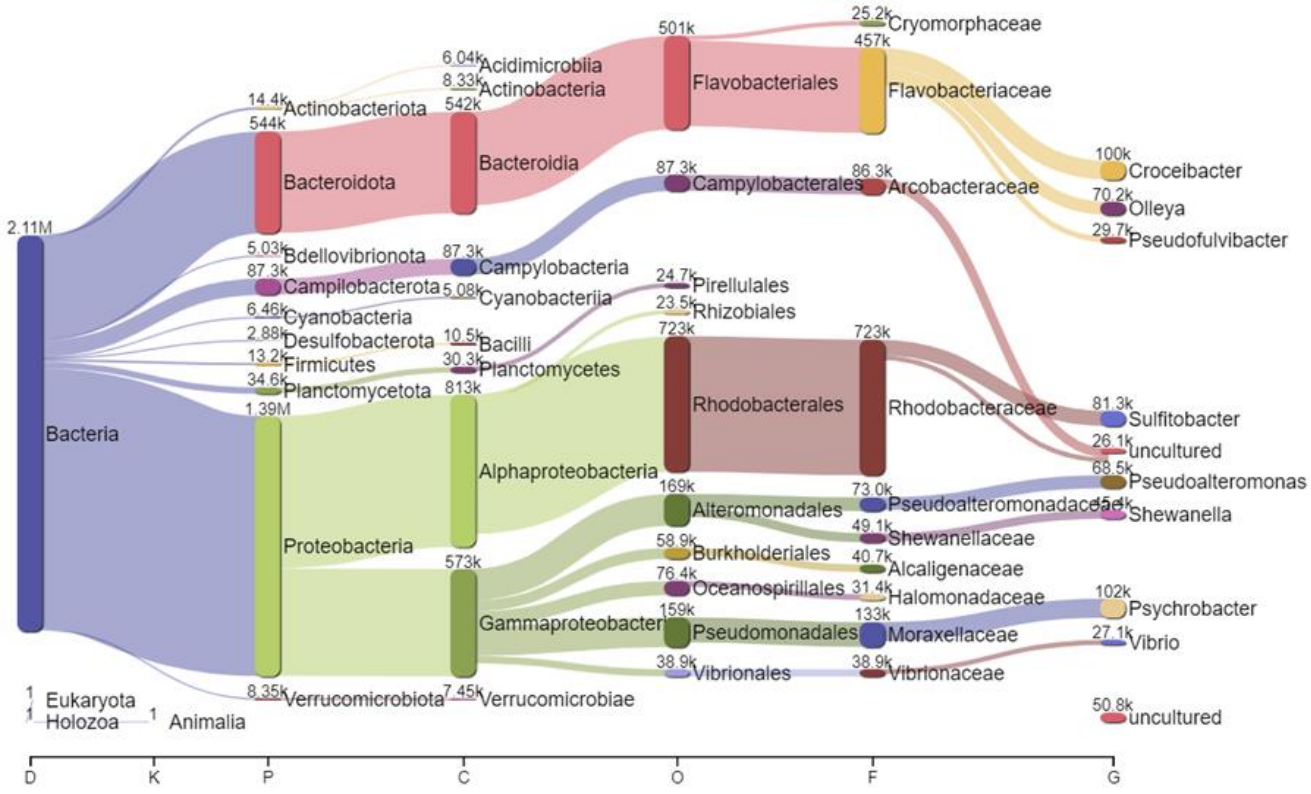


Figure 3: Taxonomic assignment of the total 2.11 million reads analysed, weighted via the proportion of reads matched to different bacterial taxonomic ranks down to genus level.

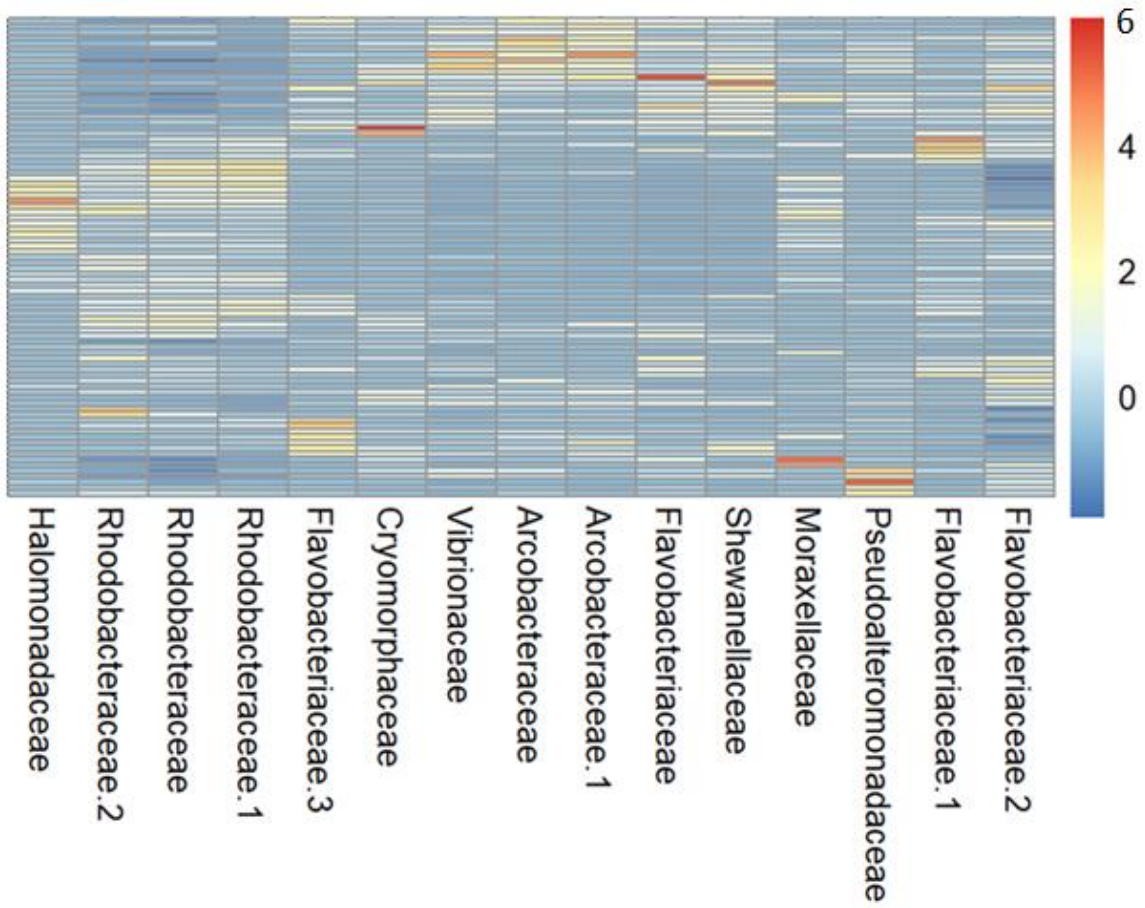


Figure 4: Heatmap showing bacterial identifications at a family level of the top 15 most relatively abundant operational taxonomic units (OTUs) within the sample set. Each row represents an individual water sample and each column a bacterial family, with some OTUs belonging to the same family.

NMDS plots were created via grouping samples according to depth at which they were collected in the water column (Figure 5a), site (Figure 5b), general bivalve larvae count category (Figure 5c), *O. edulis* larvae count category (Figure 5d) and sampling date (Figure 5e). Clear groupings are only visually apparent when samples are grouped according to date (Figure 5e). PERMANOVA statistical tests confirmed microbial differences between samples to only be statistically significant when grouped according to date ($p= 0.001$, $r^2 = 0.27415$) and also bivalve count category ($p= 0.007$, $r^2 = 0.04703$). The variable of date accounted for a much greater proportion of variance (27.4%) within the dataset, compared to the variable of bivalve count category (4.7%). The microbial compositions of samples collected at different depths ($p= 0.342$, $r^2 = 0.0216$), sites ($p= 0.95$, $r^2 = 0.06307$) or belonging to different *O. edulis*

larvae count categories (p= 0.819, r^2 = 0.01726) were not statistically significantly different from one another. Abundance data of microbial taxa across low, medium and high *O. edulis* count categories was not viable due to an unequal number of samples in each category.

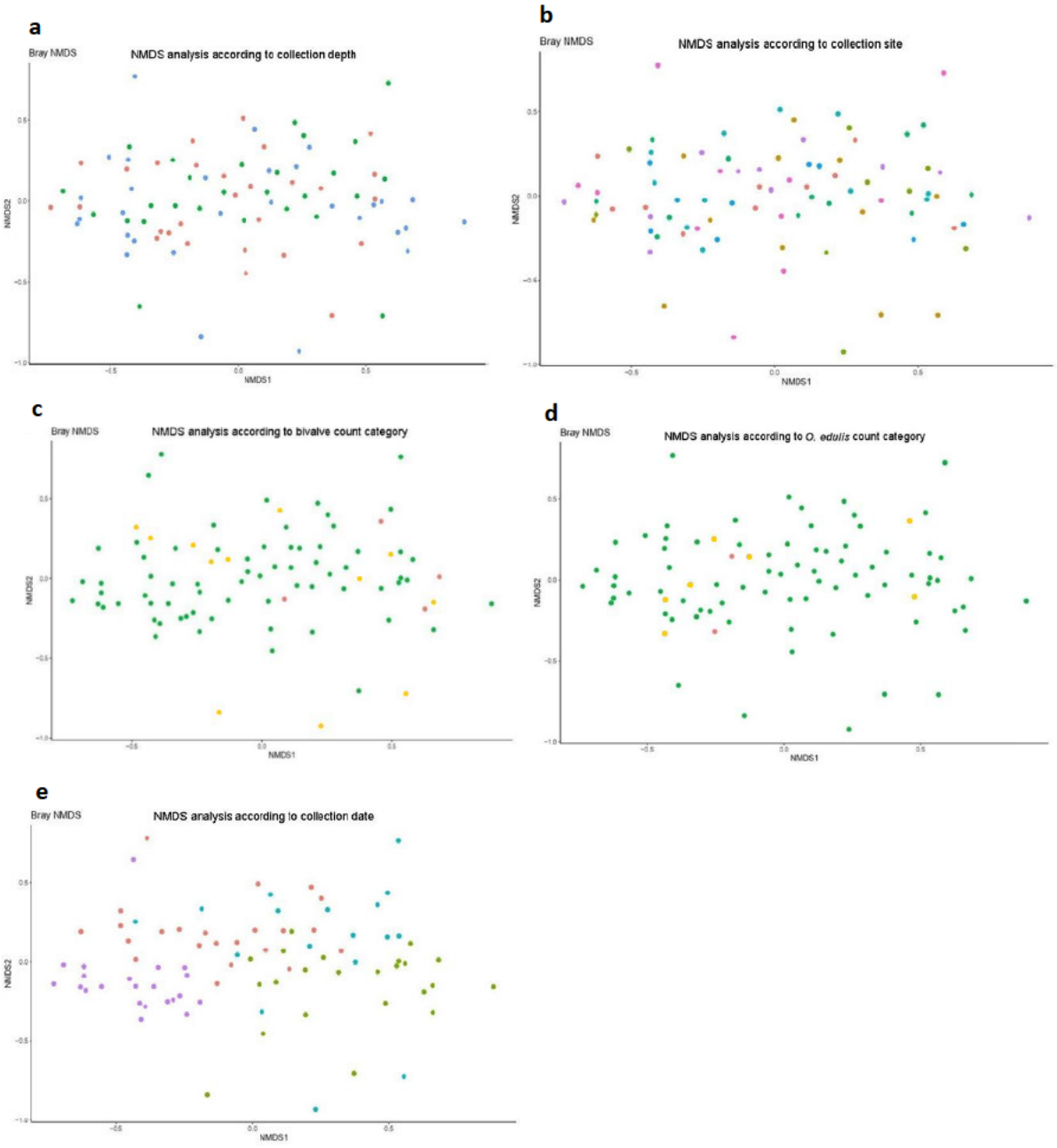


Figure 5: NMDS plots showing relationships between samples as grouped by **(a)** Depth (red = bottom, blue = surface, green = mid), **(b)** Site (red = 1, yellow = 2, moss green = 3, green = 4, turquoise = 5, blue = 6, purple = 7, pink = 8), **(c)** Mean bivalve count (red = high, green = low, blue = yellow), **(d)** Mean *O. edulis* count (red = high, green = low, blue = yellow) and **(e)** Date collected (purple = 27/06/2019, red = 11/07/2019, blue = 26/07/2019, green = 19/08/2019).

Having identified the date of collection as the most significant driver of microbial differences between samples; species richness of the microbiome throughout the spawning season was analysed (see Figure 6). Alpha diversity indices of observed (Figure 6a) and Chao 1 (Figure 6b) values were calculated for each sample, and then grouped according to their collection date. Observed alpha diversity statistics across different dates were not found to be statistically significant with a Kruskal Wallis test reporting a p value of 0.1569. Similarly, measures of Chao indices were found not significantly different between dates (p = 0.2132).

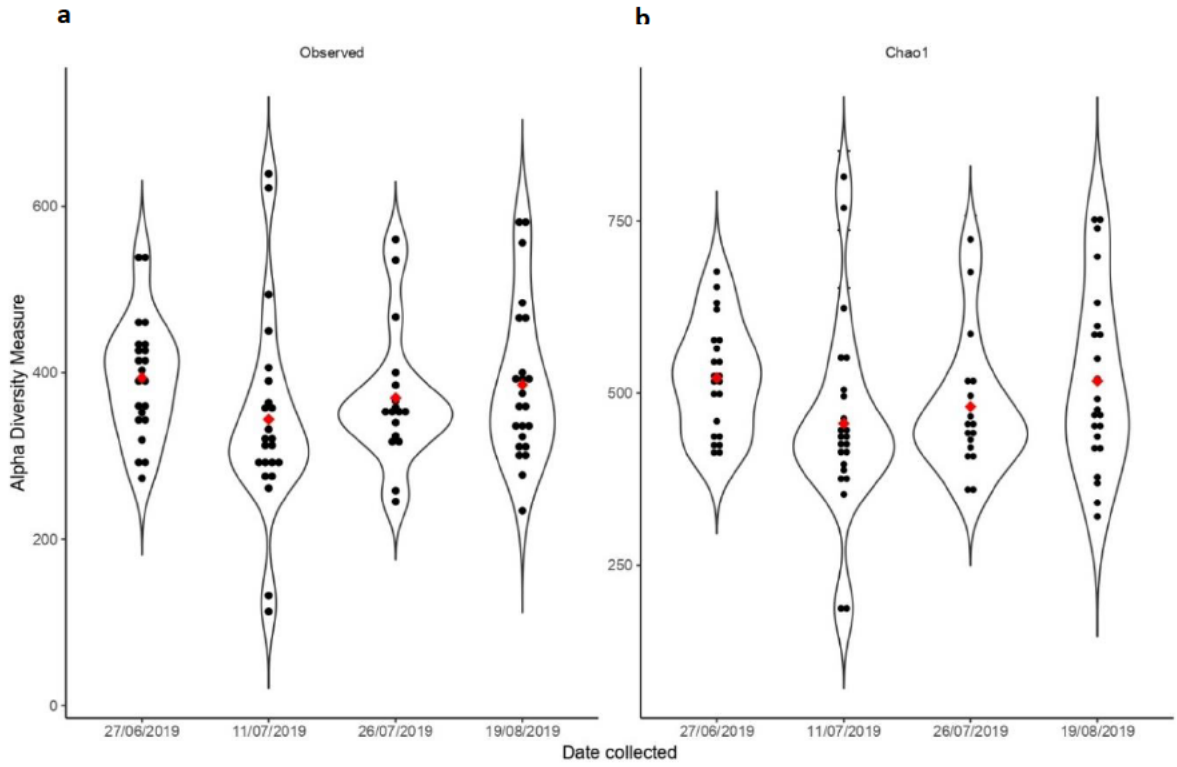


Figure 6: Alpha diversity measures of Observed (**a**) and Chao 1 (**b**) values across different dates of sample collection.

As species richness was not significantly different between samples collected on different dates, we reasoned that microbial population differences must be driven by the abundance of certain bacterial species. Figure 7 shows the relative abundance of the 9 most abundant bacterial families found in all samples for of the four sampling dates. Most notably, at the start of the spawning season on the 27/06/19 and 11/07/2019, the Rhodobacteraceae family

was especially abundant (46.9% and 46.6% of the total reads). As the spawning season progressed, this prevalence of Rhodobacteraceae decreased (24.9% and 19.6% of total reads) on 26/07/2019 and 19/08/2019.

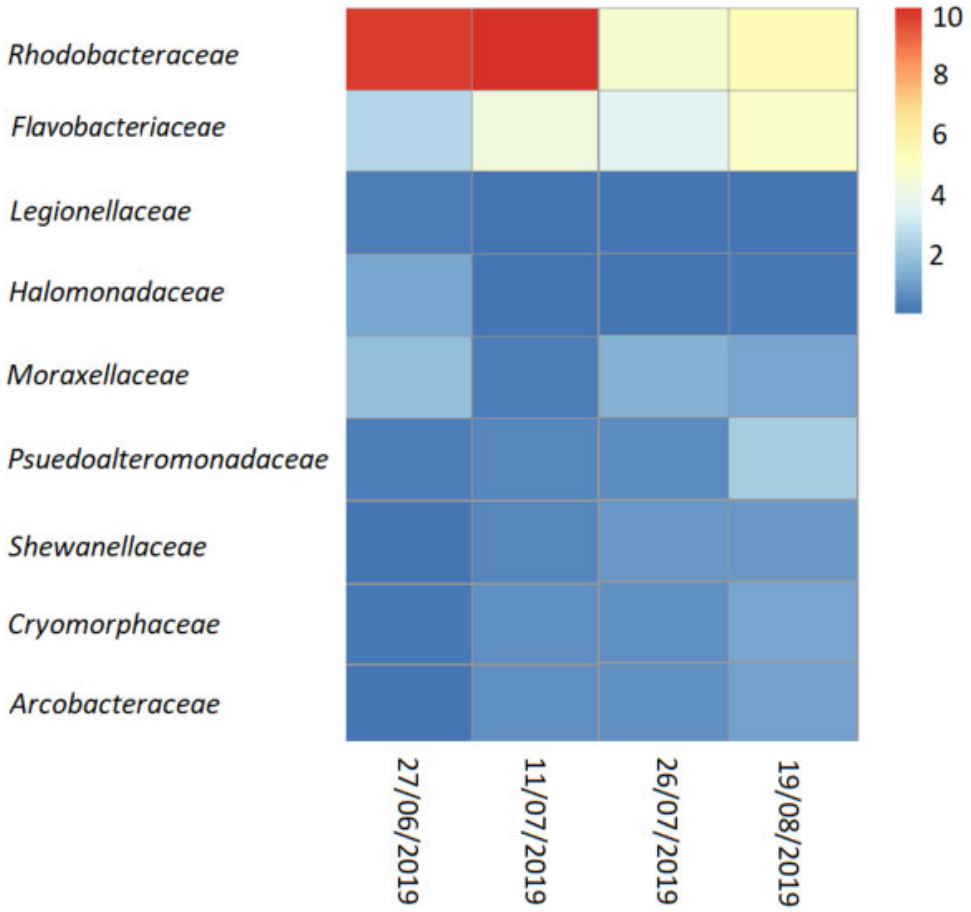


Figure 7: Distribution of the 9 most relatively abundant bacterial families throughout the spawning season.

We examined the most abundant OTUs within this *Rhodobacteraceae* family (Figure 8). The most abundant OTU belonged to an unknown genus, and appeared to drive the overall abundance profile pattern seen for the Rhodobacteraceae family – becoming less abundant as the spawning season progressed (Figure 8a). Following a similar abundance profile, the second most abundant OTU within the Rhodobacteraceae family was identified as belonging to the *Sulfitobacter* genus (Figure 8b). As the *Vibrio* genus of bacteria is of great biological importance to *O. edulis* the abundance profile of OTUs belonging to this group of bacteria throughout the spawning season was also investigated (Figure 8c). A Spearman Rank

Correlation test between the abundance of the *Vibrio* and Rhodobacteraceae throughout the spawning season reported a p value of 0.00000005359 and a rho value of – 0.5461442 indicating a statistically significant moderate negative correlation between these two taxa.

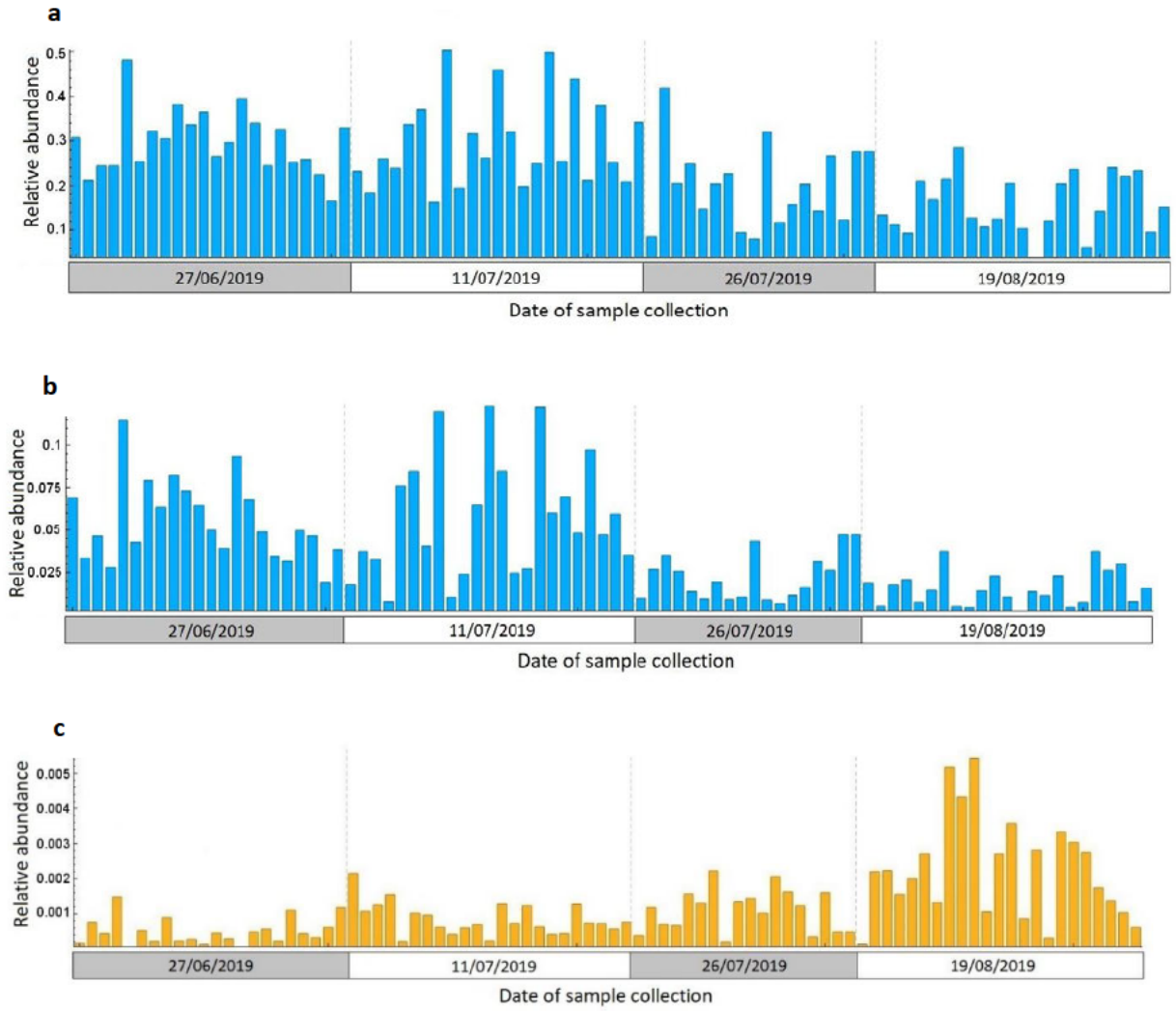


Figure 8: Relative abundance profile of the most abundant genus (uncultured) (**a**) and second most abundant genus (*Sulfitobacter*) (**b**) found within the Rhodobacteraceae family throughout the spawning season. Relative abundance profile of the *Vibrio* genus throughout the spawning season (**c**). Each bar is representative of one sample.

Discussion:

Broad-scale findings and applications:

The data presented here contributes to the existing literature defining the microbiomes of bivalve spawning environments and the larval oysters within (Arfken et al, 2021; King et al, 2019; Nguyen et al). Such information is vital in order to understand the relationships between beneficial, harmful and inconsequential bacteria and the interactions between their host and environment. This baseline data is needed in order to explore different methods of microbial manipulation within oyster farming. There is an increasing amount of research being aimed at the development of targeted therapies such as probiotics for use in aquaculture (El-Saadony et al, 2021; Gatesoupe, 1999; Subedi and Shrestha, 2020). The use of probiotic treatments within oyster farming could provide a great advantage to the industry, potentially reducing mass mortality disease events without the need for antibiotics. Such therapies function via establishing a microbial balance within the host (Sahu et al, 2008). Therefore, their development is reliant upon research such as this, which aims to increase current understanding of the baseline larval and environmental microbiomes.

We report a diverse range of bacterial taxa which changed significantly throughout the spawning season. These changes were most closely correlated with date, and we reasoned this to be driven by changes in the abundance of certain taxa - most notably, those belonging to the Rhodobacteraceae family. The influence of various abiotic factors and how they differed between collection dates is discussed below. We did not detect a microbiome signature correlated with *O. edulis* larval density (see Figure 5d), potentially suggesting that there isn't a strong larvae-associated microbiome structure. In contrast to similar studies conducted in hatchery settings (Arfken et al, 2021; Kunselman et al 2022; Timmins-Schiffman et al, 2021), we are unable to determine exactly how *O. edulis* larvae abundance contributes to this microbial structure, but our study highlights a microbial environment in which a natural spawning event of a successful oyster ecosystem takes place.

Observations of key taxa:

The high abundance of Rhodobacteraceae reported is not unexpected as this is one of the nine most widely distributed groups of bacteria found within the marine environment, estimated to make up 30% of Alphaproteobacteraceae found in the pelagic (Pohlner et al, 2019). This is a highly diverse yet understudied group of bacteria comprising an estimated 170 genera (Simon et al, 2017). The most abundant genus detected within this family (see Figure 8a), was identified as uncultured. Indeed, Pohlner et al, (2019) highlights how the majority of genera within Rhodobacteraceae remain uncultured, and suggests that most thrive under anoxic conditions and so are difficult to isolate using common cultivation-based approaches. The second most abundant genus within the Rhodobacteraceae family was identified as *Sulfitobacter* (see Figure 8b). Many different roles of *Sulfitobacter* within the marine environment have been reported including sulphur metabolism, carbon cycling (Prabagaran et al, 2007; Wu et al, 2021), biofilm formation (Beleneva, Skriptsova, and Svetashev, 2017; Ding et al, 2023) and even providing algae with protection against pathogens (Beiralas, Ozer and Segev, 2023). These are all possible roles of *Sulfitobacter* found in Loch Ryan, however a lack of taxonomic resolution to a species level makes it difficult to confirm these roles any further. Although long-read sequencing can offer species-level resolution, this was not achieved throughout this study. This limitation is attributed to gaps in the 16S reference database, where marine bacterial species are often poorly defined.

The Rhodobacteraceae and Halomonadaceae families are seen to have similar groupings within the complete sample set (see Figure 4). A high abundance of the Halomonadaceae family can be anticipated as they are known to thrive in high salinity marine environments (Haba et al, 2023). Such similar groupings could indicate that species from these families occupy similar ecological niches, however without further studies little else can be interpreted from these results. Within the current literature, there is a lack of studies which report on the *O. edulis* microbiome. It is therefore not known whether Rhodobacteraceae and Halomonadaceae families have been previously reported within the larval microbiome of this species. Rhodobacteraceae is however known to be highly abundant (47%) within the *Crassostrea gigas* larval microbiome (Asmani et al, 2016) and is a member of the *Crassostrea virginica* core larval microbiome (Arfken et al, 2021). Although the data collected here is

representative of the environmental microbiome as a whole, it could therefore be hypothesised that the detection of Rhodobacteraceae formed a part of the *O. edulis* microbiome.

Figure 8 highlights opposite abundance profiles between genera of Rhodobacteraceae and the *Vibrio* genus. It could be hypothesised that these two groups of bacteria interact with one another, due to Rhodobacteraceae having vibriostatic properties. Geng et al, (2008) has been able to prove the production of the anti-microbial compound tropodithietic acid (TDA) in marine Roseobacters (a subgroup within Rhodobacteraceae). Taking similar principles *in vivo*, Chumpol et al, (2017) reports three strains of bacteria most closely related to *Rhodobacter sphaeroides* to have antimicrobial properties against *Vibrio harveyi*, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Therefore, whilst there is not currently sufficient evidence to conclude that the abundance profiles shown in Figure 8 are directly linked to one another, investigating a hypothesis based around TDA production in the Rhodobacteraceae found could have important applications for microbial control methods in oyster hatcheries.

The influence of abiotic factors:

It is well understood that abiotic factors have a significant effect on microbiomes of the general marine environment (Wang et al, 2021), marine invertebrates (Brothers et al, 2018; Nelson et al, 2020; Ramsby et al, 2017; Song et al, 2024), and more specifically of oyster larvae (Arfken et al, 2021; King et al, 2019; Mackenzie et al, 2022). Chapman et al, (2021) collected a range of different physiochemical parameters alongside sample collection, including daily averages of water salinity and temperature sum. ANOVA statistical tests concluded that there was no significant difference between mean water temperatures ($p= 0.736$) or salinity measurements ($p = 0.713$) taken across the different sampling dates. Microbial changes throughout the spawning season are therefore unlikely to be driven by these variables. Chapman et al, (2021) also took into account variabilities in the tide of Loch Ryan by recording day in the lunar cycle throughout the sampling period. Tidal range varied with sampling dates ranging between 9 and 24 days since the new moon. Since no significant difference was found in the microbiome of samples collected at different points within the water column (see Figure 5a), it is also unlikely that microbial differences were driven by variations in the tide.

Future recommendations:

Limitations of this study can be used to provide improvements and recommendations for similar future works. When interpreting the data presented here, it is key to consider that the sampling process allowed for microbial characterisation of all material condensed on the 90 µm mesh, and not exclusively of *O. edulis* larvae. This is a limitation of conducting a study throughout a natural spawning event, where practically, *O. edulis* larvae cannot be separated from the larvae of other species and organic matter. In contrast, studies conducted in a hatchery environment would be able to allow for a sampling process which is fully representative of the *O. edulis* microbiome. Such studies would be highly beneficial in allowing for a comparison of OTUs to those found with natural settings such as Loch Ryan – but are currently lacking from the current literature.

We discuss possible relationships between key taxa, however in order to understand these further, taxonomic identification down to a species level is required. In the case of some taxa such as those belonging to the *Vibrio* genera, a 16S – based approach does not provide sufficient taxonomic resolution to identify down to a species level. Future works into determining the relationship between members of the Rhodobacteraceae family and species of *Vibrio* pathogenic to oysters would also be of great importance. Such studies may benefit from whole genome sequencing (WGS) of bacterial isolates of interest in order to give the best possible chance of species identification. This would aid in developing targeted probiotic strategies and minimise the effects of mass mortality events in commercial hatcheries. Additionally, it may provide extra resolution to separate the bivalve larvae from the environmental (water) fraction and analyse these separately.

Conclusions:

This study provides unique data, in outlining the environmental microbiome during a natural *O. edulis* spawning event. We report that microbial differences throughout the spawning season were most closely correlated with date, and not site, larval count category or depth of sampling within the water column. In contrast to what was hypothesised, a strong microbial signature was not associated with the larvae sampled. The data presented

improves current understanding of the *O. edulis* microbiome and allows for recommendations on future studies to improve cultivation strategies.

References:

Allison, S., Hardy, M., Hayward, K., Cameron, T. C., & Underwood, G. J. C. (2020). Strongholds of *Ostrea edulis* populations in estuaries in Essex, SE England and their association with traditional oyster aquaculture: Evidence to support a MPA designation. *Journal of the Marine Biological Association of the United Kingdom*, *100*(1), 27–36. https://doi.org/10.1017/S0025315419001048

Arfken, A., Song, B., Allen, S. K., & Carnegie, R. B. (2021). Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. *Aquaculture*, *531*. https://doi.org/10.1016/j.aquaculture.2020.735955

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*, 464, 434-444.

Beiralas, R., Ozer, N., & Segev, E. (2023). Abundant Sulfitobacter marine bacteria protect *Emiliania huxleyi* algae from pathogenic bacteria. *ISME Communications*, *3*(1). https://doi.org/10.1038/s43705-023-00311-y

Beleneva, I. A., Skriptsova, A. V., & Svetashev, V. I. (2017). Characterization of biofilm-forming marine bacteria and their effect on attachment and germination of algal spores. *Microbiology (Russian Federation)*, *86*(3), 317–329. https://doi.org/10.1134/S0026261717030031

Boutet, I., Alves Monteiro, H. J., Baudry, L., Takeuchi, T., Bonnivard, E., Billoud, B., Farhat, S., Gonzales-Araya, R., Salaun, B., Andersen, A. C., Toullec, J. Y., Lallier, F. H., Flot, J. F., Guiglielmoni, N., Guo, X., Li, C., Allam, B., Pales-Espinosa, E., Hemmer-Hansen, J., … Tanguy, A. (2022). Chromosomal assembly of the flat oyster (*Ostrea edulis* L.) genome as a new genetic resource for aquaculture. *Evolutionary Applications*, *15*(11), 1730–1748. https://doi.org/10.1111/eva.13462

Breitwieser, F. P., & Salzberg, S. L. (2020). Pavian: Interactive analysis of metagenomics data for microbiome studies and pathogen identification. *Bioinformatics*, *36*(4), 1303–1304. https://doi.org/10.1093/bioinformatics/btz715

Brothers, C. J., Van Der Pol, W. J., Morrow, C. D., Hakim, J. A., Koo, H., & McClintock, J. B. (2018). Ocean warming alters predicted microbiome functionality in a common sea urchin. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1881). https://doi.org/10.1098/rspb.2018.0340

Chapman, E., Perez, A. R., Board, E. M., Hugh-jones, T., Bromley, C., James, M., Diele, K., & Sanderson W, G. (2021). Optimising recruitment in habitat creation for the native European oyster (*Ostrea edulis*): Implications of temporal and spatial variability in larval abundance. *Marine Pollution Bulletin* (170), 112579. https://doi.org/10.1016/j.marpolbul.2021.112579

Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T., & Kanzaki, H. (2017). In vitro and in vivo selection of probiotic purple nonsulphur bacteria with an ability

to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other *vibrios*. *Aquaculture Research*, *48*(6), 3182–3197. https://doi.org/10.1111/are.13149

Cole, H. A. (1941). The fecundity of *Ostrea edulis*. *Journal of the Marine Biological Association of the United Kingdom*, 25(2), 243-260.

Colsoul, B., Boudry, P., Pérez-Parallé, M. L., Bratoš Cetinić, A., Hugh-Jones, T., Arzul, I., Mérou, N., Wegner, K. M., Peter, C., Merk, V., & Pogoda, B. (2021). Sustainable large-scale production of European flat oyster (*Ostrea edulis*) seed for ecological restoration and aquaculture: a review. *Reviews in Aquaculture*, *13*(3), 1423–1468. https://doi.org/10.1111/raq.12529

De Coster, W., D'Hert, S., Schultz, D. T., Cruts, M., & Van Broeckhoven, C. (2018). NanoPack: Visualizing and processing long-read sequencing data. *Bioinformatics*, *34*(15), 2666–2669. https://doi.org/10.1093/bioinformatics/bty149

Ding, W., Wang, S., Qin, P., Fan, S., Su, X., Cai, P., Lu, J., Cui, H., Wang, M., Shu, Y., Wang, Y., Fu, H. H., Zhang, Y. Z., Li, Y. X., & Zhang, W. (2023). Anaerobic thiosulfate oxidation by the Roseobacter group is prevalent in marine biofilms. *Nature Communications*, *14*(1). https://doi.org/10.1038/s41467-023-37759-4

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A. O., Dhama, K., & Abdel-Latif, H. M. R. (2021). The functionality of probiotics in aquaculture: An overview. In *Fish and Shellfish Immunology* (Vol. 117, pp. 36–52). Academic Press. https://doi.org/10.1016/j.fsi.2021.07.007

Engelsma, M. Y., Kerkhoff, S., Roozenburg, I., Haenen, O. L. M., Van Gool, A., Sistermans, W., Wijnhoven, S., & Hummel, H. (2010). Epidemiology of *Bonamia ostreae* infecting European flat Oysters *Ostrea edulis* from Lake Grevelingen, The Netherlands. *Marine Ecology Progress Series*, *409*, 131–142. https://doi.org/10.3354/meps08594

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. In *Aquaculture* (Vol. 180). www.elsevier.nlrlocateraqua-online

Geng, H., Bruhn, J. B., Nielsen, K. F., Gram, L., & Belas, R. (2008). Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. *Applied and Environmental Microbiology*, *74*(5), 1535–1545. https://doi.org/10.1128/AEM.02339-07

Guy, C., Smyth, D., & Roberts, D. (2019). The importance of population density and inter-individual distance in conserving the European oyster *Ostrea edulis*. *Journal of the Marine Biological Association of the United Kingdom*, *99*(3), 587–593. https://doi.org/10.1017/S0025315418000395

Hayer S, Bick A, Brandt A, Ewers-Saucedo C, Fiege D, Füting S, Krause-Kyora B, Michalik P, Reinicke GB, Brandis D. (2019). Coming and going–Historical distributions of the European oyster *Ostrea edulis Linnaeus*, 1758 and the introduced slipper limpet *Crepidula fornicata Linnaeus*, 1758 in the North Sea. *PLoS One*, 14(10), e0224249.

Hughes, A., Bonačić, K., Cameron, T., Collins, K., da Costa, F., Debney, A., van Duren, L., Elzinga, J., Fariñas-Franco, J. M., Gamble, C., Helmer, L., Holbrook, Z., Holden, E., Knight, K., Murphy, J. A. J., Pogoda, B., Pouvreau, S., Preston, J., Reid, A., … zu Ermgassen, P. S. E. (2023). Site selection for European native oyster (*Ostrea edulis*) habitat restoration projects: An expert-derived consensus. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *33*(7), 721–736. https://doi.org/10.1002/aqc.3917

Joyce, A., Holthuis, T. D., Charrier, G., & Lindegarth, S. (2013). Experimental effects of temperature 446 and photoperiod on synchrony of gametogenesis and sex ratio in the European oyster 447 *Ostrea edulis* (*Linnaeus*). *Journal of Shellfish Research*, 32, 447-458.

Kennon, N.A., Robertson-Jones, A., Jemmett, S., Hugh-Jones, T., Bell, M.C. and Sanderson, W.G., 2023. Rotational fishing enables biodiversity recovery and provides a model for oyster (*Ostrea edulis*) habitat restoration. *PloS one*, 18(3), p.e0283345.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS ONE*, *7*(12). https://doi.org/10.1371/journal.pone.0051475

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

Kunselman, E., Minich, J. J., Horwith, M., Gilbert, J. A., & Allen, E. E. (2022). Variation in Survival and Gut Microbiome Composition of Hatchery-Grown Native Oysters at Various Locations within the Puget Sound. *Microbiology Spectrum*, *10*(3). https://doi.org/10.1128/spectrum.01982-21

Li, Q., Wang, Q., Liu, S., & Kong, L. (2011). Selection response and realized heritability for growth in three stocks of the Pacific oyster *Crassostrea gigas*. *Fisheries Science*, *77*(4), 643–648. https://doi.org/10.1007/s12562-011-0369-0

Mackenzie, C. L., Pearce, C. M., Leduc, S., Roth, D., Kellogg, C. T. E., Clemente-Carvalho, R. B. G., & Green, T. J. (2022). Impacts of Seawater pH Buffering on the Larval Microbiome and Carry-Over Effects on Later-Life Disease Susceptibility in Pacific Oysters. *Applied and Environmental Microbiology*, *88*(22). https://doi.org/10.1128/aem.01654-22

Martin, M. (n.d.). *TECHNICAL NOTES*. http://www-huber.embl.de/users/an-

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0061217

Nelson, K. S., Baltar, F., Lamare, M. D., & Morales, S. E. (2020). Ocean acidification affects microbial community and invertebrate settlement on biofilms. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-60023-4

Nguyen, V. K., King, W. L., Siboni, N., Mahbub, K. R., Dove, M., O'Connor, W., Seymour, J. R., & Labbate, M. (2020). The Sydney rock oyster microbiota is influenced by location, season and genetics. *Aquaculture*, *527*. https://doi.org/10.1016/j.aquaculture.2020.735472

Pohlner, M., Dlugosch, L., Wemheuer, B., Mills, H., Engelen, B., & Reese, B. K. (2019). The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. *Frontiers in Microbiology*, *10*(APR). https://doi.org/10.3389/fmicb.2019.00659

Pouvreau, S., Lapègue, S., Arzul, I., & Boudry, P. (2023). Fifty years of research to counter the decline of the European flat oyster (Ostrea edulis): A review of French achievements and prospects for the restoration of remaining beds and revival of aquaculture production. In *Aquatic Living Resources* (Vol. 36). EDP Sciences. https://doi.org/10.1051/alr/2023006

Prabagaran, S. R., Manorama, R., Delille, D., & Shivaji, S. (2007). Predominance of Roseobacter, Sulfitobacter, Glaciecola and Psychrobacter in seawater collected off Ushuaia, Argentina, Sub-Antarctica. *FEMS Microbiology Ecology*, *59*(2), 342–355. https://doi.org/10.1111/j.1574-6941.2006.00213.x

Ramsby, B. D., Hoogenboom, M. O., Whalan, S., & Webster, N. S. (2018). Elevated seawater temperature disrupts the microbiome of an ecologically important bioeroding sponge. *Molecular Ecology*, *27*(8), 2124–2137. https://doi.org/10.1111/mec.14544

Sahu, M. K., Swarnakumar, N. S., Sivakumar, K., Thangaradjou, T., & Kannan, L. (2008). Probiotics in aquaculture: Importance and future perspectives. In *Indian Journal of Microbiology*, 4(3), 299–308. https://doi.org/10.1007/s12088-008-0024-3

Šegvić-Bubić, T., Žužul, I., Talijančić, I., Ugrin, N., Lepen Pleić, I., Žuvić, L., Stagličić, N., & Grubišić, L. (2020). Translocation and Aquaculture Impact on Genetic Diversity and Composition of Wild Self-Sustainable Ostrea edulis Populations in the Adriatic Sea. *Frontiers in Marine Science*, *7*. https://doi.org/10.3389/fmars.2020.00084

Simon, M., Scheuner, C., Meier-Kolthoff, J. P., Brinkhoff, T., Wagner-Döbler, I., Ulbrich, M., Klenk, H. P., Schomburg, D., Petersen, J., & Göker, M. (2017). Phylogenomics of Rhodobacteraceae reveals evolutionary adaptation to marine and non-marine habitats. *ISME Journal*, *11*(6), 1483–1499. https://doi.org/10.1038/ismej.2016.198

Smyth, D., Kregting, L., Elsäßer, B., Kennedy, R., & Roberts, D. (2016). Using particle dispersal models to assist in the conservation and recovery of the overexploited native oyster (*Ostrea edulis*) in an enclosed sea lough. *Journal of Sea Research*, *108*, 50–59. https://doi.org/10.1016/j.seares.2015.12.009

Song, Z., Li, K., & Li, K. (2024). Acute effects of the environmental probiotics Rhodobacter sphaeroides on intestinal bacteria and transcriptome in shrimp *Penaeus vannamei*. *Fish and Shellfish Immunology*, *145*. https://doi.org/10.1016/j.fsi.2023.109316

Thomas, S., Collins, K., Hauton, C., & Jensen, A. (2022). A Review of the Ecosystem Services Provided by the Native Oyster (*Ostrea edulis*): Implications for Restoration. *IOP Conference*

Series: Materials Science and Engineering, *1245*(1), 012010. https://doi.org/10.1088/1757-899x/1245/1/012010

Thurstan, R. H., Hawkins, J. P., Raby, L., & Roberts, C. M. (2013). Oyster (*Ostrea edulis*) extirpation and ecosystem transformation in the Firth of Forth, Scotland. *Journal for Nature Conservation*, *21*(5), 253–261. https://doi.org/10.1016/j.jnc.2013.01.004

Timmins-Schiffman, E., White, S. J., Thompson, R. E., Vadopalas, B., Eudeline, B., Nunn, B. L., & Roberts, S. B. (2021). Coupled microbiome analyses highlights relative functional roles of bacteria in a bivalve hatchery. *Environmental Microbiomes*, *16*(1). https://doi.org/10.1186/s40793-021-00376-z

Wang, Z., Tsementzi, D., Williams, T. C., Juarez, D. L., Blinebry, S. K., Garcia, N. S., Sienkiewicz, B. K., Konstantinidis, K. T., Johnson, Z. I., & Hunt, D. E. (2021). Environmental stability impacts the differential sensitivity of marine microbiomes to increases in temperature and acidity. *ISME Journal*, *15*(1), 19–28. https://doi.org/10.1038/s41396-020-00748-2

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Completing bacterial genome assemblies with multiplex MinION sequencing. *Microbial Genomics*, *3*(10). https://doi.org/10.1099/mgen.0.000132

Wickham, M. H. (2014). *Package "ggplot2" Type Package Title An implementation of the Grammar of Graphics*.

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*(1). https://doi.org/10.1186/s13059-019-1891-0

Wood, D. E., & Salzberg, S. L. (2014). *Kraken: ultrafast metagenomic sequence classification using exact alignments*. http://ccb.jhu.edu/software/kraken/.

Wu, B., Liu, F., Fang, W., Yang, T., Chen, G. H., He, Z., & Wang, S. (2021). Microbial sulfur metabolism and environmental implications. In *Science of the Total Environment* (Vol. 778). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2021.146085

Data Availability Statement

The data that support the findings of this study are available in Dryad Digital Repository at https://doi.org/10.5061/dryad.83bk3jb1s. This includes abundance, taxonomic and metadata tables used for the analysis of microbial information from Loch Ryan water samples. This Dyrad data submission gives a detailed account of metadata variable used, sample collection and data processing methods. The reviewer sharing link provided by Dryad is available at:
https://datadryad.org/stash/share/K38H7bFkJGuwpI80uouQsLu_tyaZgcbKlja81x9_KGU

Author Contributions

Conceptualization, H.F., T.R., T.P.B. and E.C.; methodology, H.F, T.P.B., T.R, F.M.B., E.C.N.C, W.G.S., K.D., A.R.P., and T.H.J.; investigation, H.F., T.R., T.P.B., E.C.N.C., A.R.P. and F.M.B; data curation, H.F., T.R., T.P.B and E.C.N.C.; writing—original draft preparation, H.F., T.R and T.P.B.; writing—review and editing, H.F., T.R., E.C.N.C., A.R.P., T.H.J., K.D., W.G.S., F.M.P and T.P.B; funding acquisition, T.P.B., W.G.S and T.H.J. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Chapter three: A microbiome investigation throughout different spawning events of UK oyster hatcheries



Abstract:

Improving the current understanding of the larval microbiome in commercial settings is vital in order to implement findings from laboratory studies, improve seedstock production in hatcheries, and secure the sustainability of the industry. Samples of larvae and the hatchery environments in which they were raised were collected throughout two oyster spawning events at UK hatcheries – *Crassostrea gigas* at Morecambe Bay oyster hatchery and *Ostrea edulis* at Portsmouth University hatchery and used for full-length 16S rRNA sequencing. Core microbial taxa were identified from larvae at both hatcheries. This, along with reducing species richness over time at Portsmouth, suggests that the larval microbiome stabilises in later developmental stages, possibly less easily influenced by environmental microbes. Microbiomes of different sample types were significantly different from one another, with larval microbiomes being most closely associated with those of either pallial cavity water (Portsmouth), suggesting a source of the core microbes for *O. edulis*, or biofilm (Morecambe Bay), suggesting either a similar niche, or a source or sink of the *C. gigas* larval microbiome. Microbial implications of hatchery practices were also investigated with successive water filtration methods at both hatcheries concluded to be successful in progressively reducing microbial content within the water, alongside their standard role of reducing microbial load. This chapter contributes to the understanding of the larval microbiome in a setting which is directly applicable to their commercial production.

Introduction:

Oyster farming is a key sector of UK aquaculture:

Oysters are the most commonly produced mollusc by quantity (Botta et al, 2020), with their aquaculture possessing great untapped potential to provide sustainable global protein sources. *Crassostrea gigas*, also known as *Magallana gigas* or the Pacific oyster, is the most commonly produced UK oyster species and is a sustainable source of protein (Humphreys et al, 2014). The UK oyster industry also produces *Ostrea edulis* (the European flat oyster), for a relatively minor farming interest and for growing applications in ecological restoration (Colsoul et al, 2021), alongside. The UK's production of *C. gigas* outweighs that of *O. edulis* by an order of magnitude, with an estimated 67% of *C. gigas* livestock then being exported overseas (Humphreys et al, 2014). Oyster farming therefore represents a key sector of both UK and global aquaculture, providing economic activity through primary production as well as contributing to food security and environmental objectives.

Microbiomes in oyster aquaculture hatcheries:

The vast majority of farmed and restored UK oysters are spawned in oyster hatcheries before being moved into the field. Previous research into microbes associated with aquatic species, focusses on farmed species, including both fish (Lorgen-Ritchie et al, 2021; Perry et al, 2020; Uren Webster et al, 2021) and shellfish (Chen et al, 2017; Pimentel et al, 2021; Unzueta-Martínez et al, 2022a; Unzueta-Martínez et al, 2022b). Only a small proportion of this research investigates the microbiomes of the hatchery environments of these species. In the case of oyster aquaculture where the larval microbiome is so extrinsically linked with its external environment (King et al, 2019), an understanding of the hatchery microbiome is likely to be key to improving larval production and quality.

It is has been previously described that location has a significant effect on the oyster microbiome (Kunselman et al, 2022). In the adult *Ostrea lurida* (Olympia oyster), the gut microbiome has been found to significantly differ across four different locations in the Puget Sound. This was shown with oysters from some locations having a higher relative abundance of *Vibrio* in comparison to others and is hypothesised to be driven by statistically significant differences in temperature and dissolved oxygen between sites. Considering this evidence, it

could be suggested that a location-dependent effect may influence microbial variation seen within commercial oyster hatcheries. Baseline microbial studies within commercial settings are however limited within the current literature with exceptions of Arfken et al, 2021. Arfken et al, 2021 concluded that microbial variations in *Crassostrea virginica* larvae were influenced more so by hatchery type (commercial vs research) than spawning season. Although core taxa remained similar between each of the sites, commercial hatcheries displayed higher relative abundances of Mycoplasma and Bacilli in comparison to the research hatcheries. These differences are hypothesised to be attributed to differences in location, water filtration and feeding practices between the four sites investigated (Arfken et al, 2021). This highlights some of the effect of hatchery practices to the larval microbiome but leaves some doubt as to what the impact is, and how it can be exploited for improved larval production. This work also only encompassed study of the oyster larvae and the water within which they were cultured, leaving the impact of all other aspect of hatchery systems to be studied.

Disease is widely cited as the main limiting factor to oyster aquaculture and this has been acknowledged within the UK market since 1951 (Botta et al, 2020; Cole, 1949; Pernet et al, 2016). In the UK, where the oyster industry is heavily reliant upon producing a consistent and reliable supply of good quality seedstock, mass mortality events during the larval stages of the life cycle remain a major hinderance. Many of these mortalities result from disease caused by bacterial pathogens such as those belonging to the *Vibrio* genus (Barbosa Solomieu et al, 2015). Understanding the bacterial profiles of larvae and their environments during hatchery spawning events are therefore likely to be key to understanding and preventing future disease events.

Basic oyster hatchery infrastructure:

Although oysters are farmed in a vast range of different environments (Botta et al, 2020), all oyster hatcheries fulfil common criteria necessary for efficient production. Firstly, a hatchery must have sufficient space to rear oysters through three main stages of their life cycle; planktonic, planktonic free swimming and sessile (Vogeler et al, 2016). These life stages require different specialised set-ups, such as upwelling tanks for larval production and outdoor rafts or baskets for spat to fully mature (Figure 1). Oyster hatcheries also require a supply of seawater and are therefore commonly situated close to the coast. Before being used

within the hatchery, seawater is usually passed through cartridge and UV (ultraviolet) filters in order to remove debris and any potentially harmful microorganisms (Ford et al, 2001; Jorquera et al, 2002). This objective is also commonly achieved via continuous flow-through pasteurisation as practiced by Morecambe Bay and Guernsey Sea Farms in the UK. Oyster hatcheries must also have means of producing consistent supplies of live microalgae in order to condition broodstock and feed larvae and growing oysters. Algal cultures are frequently produced in high volumes which are directly fed to larvae and spat to sustain their growth and reach grow-out size (Brown and Blackburn, 2013; McCausland et al, 1999); (Figure 1). There is a trend for newer oyster hatcheries to attempt to operate as extremely clean, almost sterile, environments. However, there is a huge amount of variation in hatchery infrastructure and there has been a great deal of success in older hatcheries which work in more open systems, likely with a significant environmental influence on the conditions and microbial environment in which oysters are grown.

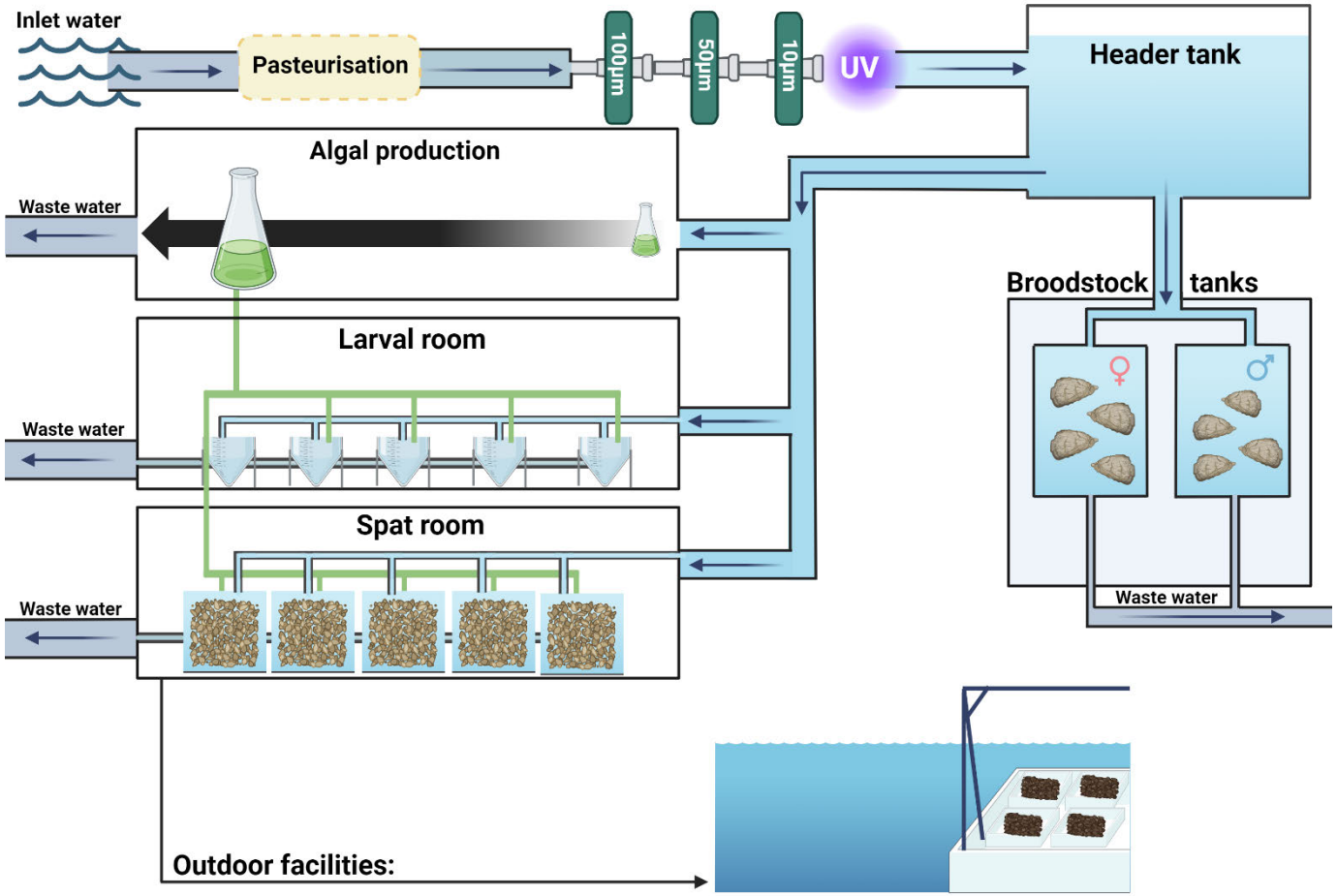


Figure 1: Schematic showing the generalised structure of an oyster hatchery. Seawater is sterilised through pasteurisation, cartridge filters and UV exposure before being used in algal, broodstock, larval and spat cultures. Larvae that mature into spat are placed into outdoor nursery facilities such as flupsy (floating upwelling systems) to grow until they are suitable for sale and grow-out on farm sites. Live microalgae are produced on mass on site and fed directly to larval, spat and adult stages. Created in BioRender.com.

Oyster production within hatcheries:

To begin a spawning event, hatcheries must first condition adult oysters to produce male and female 'broodstock' animals with mature gametes that are ready to spawn. Depending on the oyster species and environment, this conditioning process involves holding select oysters in specific temperature and dietary conditions (Adams et al, 2013; Utting and Millican, 1997) which provide the nutrition required for gametogenesis. Hatcheries then collect the gametes from the oysters via a process of 'natural' or 'strip' spawning. In the case of a strip spawning, gametes are extracted manually from broodstock oysters by creating incisions in the gonad with a sterile blade and washing out the gametes with filtered seawater. The eggs and sperm are then combined in specific ratios (circa 1 egg per 50-200 sperm) in seawater in conditions amenable for fertilisation (Allen and Buskek, 1992; Nowland et al, 2021). Conversely, animals can be induced to release gametes into the water in a more natural way, for example through temperature shock, chemical induction, or through the process of exposing ripe animals to gametes from another spawned individual (Figure 2); (Nowland et al, 2021).



Figure 2: An example of gamete release into the water column from mature spawning oysters. This image is provided by the University of Maryland Centre for Environmental Science's (UMCES) Horn Point Laboratory available at: https://mississippiriverdelta.org/oysters-101/

Spawning and development of C. gigas:

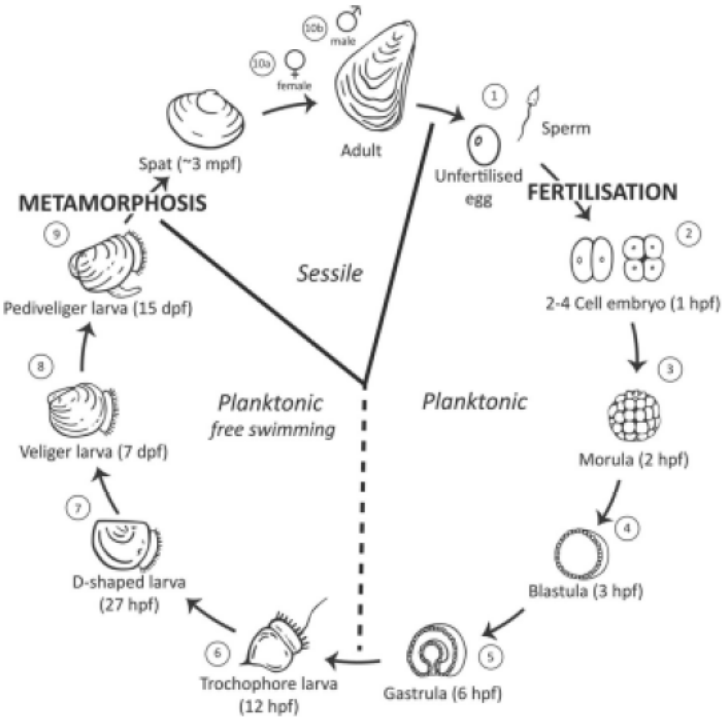
In the case of *C. gigas*, development occurs quickly and the first free swimming larval stage of the oyster, the trochophore, develops around 12 hours post fertilisation. These larvae swim freely within the water column, feeding on algae and continuing to develop into D-shaped larvae 27 hours post fertilisation. D-shaped larvae have an early bivalve shell, which forms in a characteristic 'D' shape around the soft tissue of the animal. These larvae then progress through veliger and pediveliger larval stages at seven and 15-days post fertilisation (Vogeler et al, 2016); (see Figure 3a). These hatchery timings are optimal and are generally shorter than is observed in the wild, or when conditions in the hatchery are less than optimal. The pediveliger stage is the final free-swimming stage during which larvae are preparing to undergo metamorphosis into sessile animals. In hatchery scenarios metamorphosis can either be allowed to happen naturally over time, or can be induced with epinephrine treatment (Coon et al, 1990). During metamorphosis, pediveliger larvae lose their foot and velum (a ciliated structure used for swimming and feeding); (Lucas et al, 2019). They then undergo a process also known as settlement, attachment or culching, onto a substrate and begin to develop adult shells. At this point, they start being referred to as 'spat' or 'seedstock' and are in the sessile stage of the oyster life cycle. Pacific oysters only go through a culching process once in life, and in hatchery scenarios they are generally rapidly removed from their settlement substrate, either by washing or with a fine blade, becoming what is commonly known as 'cultchless' spat. i.e., metamorphosed oysters which are not attached to substate. Cultchless spat are often preferable as they are more amenable to manipulation in farm environments than cultched spat. Hatcheries of other closely related species, and also some *C. gigas* systems across the world, use similar processes to the above but with slight alterations. For example, traditional *C. gigas* culture in parts of China and France sees spat settled directly onto stakes, which are then planted into the field for outgrowing.

Spawning and development of O. edulis:

In comparison to *C. gigas*, *O. edulis* has a very different reproductive process. In favourable environmental conditions, ripe male individuals release sperm into the water column where it can be distributed by currents. During water filtration, female oysters take in this sperm

into the mantle cavity to mix with eggs, resulting in fertilisation. After fertilisation, the female oyster will brood larvae within the mantle cavity where they into trochophore larvae and then into 'D – shaped' veliger larvae. After 7-10 days of being held within the mantle cavity, larvae are released into the water column to continue the rest of their life cycle (Helmer et al, 2019); (Figure 3b).

a.



b.

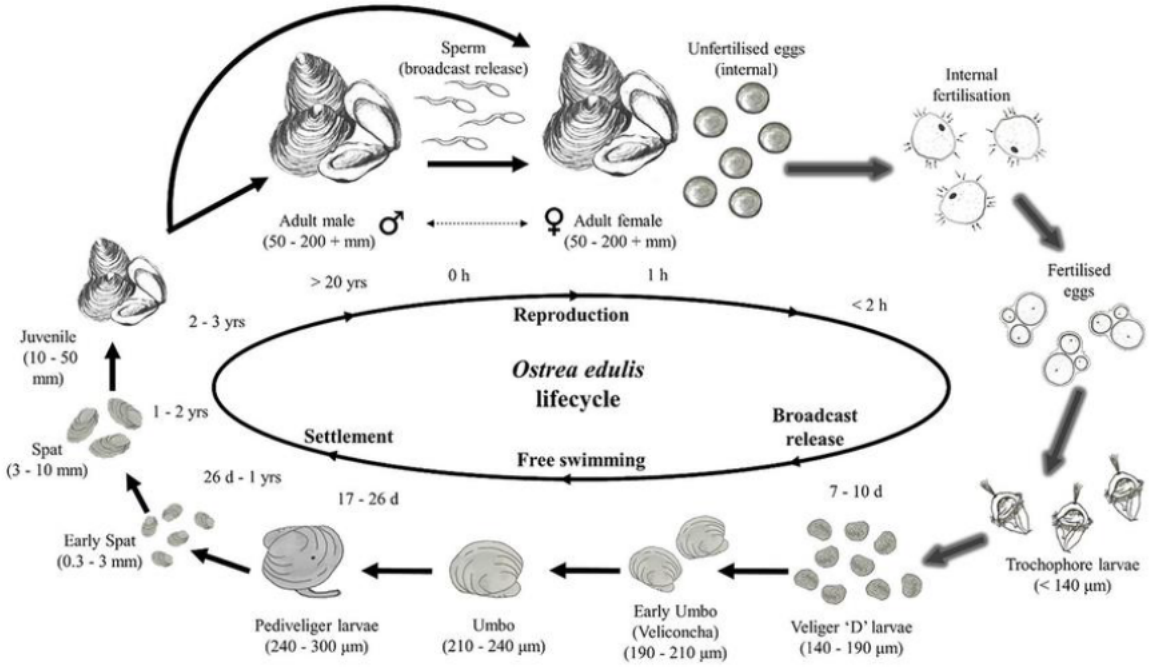


Figure 3: (a) The life cycle of Crassostrea gigas, where 'hpf' = hours post fertilisation, 'dpf'= days post fertilisation and 'mpf'=months post fertilisation. Figure taken from Vogeler et al, 2016. **(b)** The life cycle of *Ostrea edulis.* Glow effect arrows are used to represent stages that occur internally within the female mantle cavity, whilst plain arrows indicate external life stages. Figure taken from Helmer et al, 2019.

The production of Ostrea edulis at Portsmouth University

This chapter covers a microbiome study of a naturally induced *O. edulis* spawning in the hatchery of the Portsmouth University Institute of Marine Science facilities (IMS). This oyster hatchery is based in the South coast of England (see Figure 4), where the seawater is supplied directly from within Langstone harbour. This facility operates under research and restoration-based objectives, producing *O. edulis* for ecological restoration efforts in the Solent.

In summary, within this hatchery, broodstock are maintained in eight different tanks which feed directly into four larval collection trays. Upon internal fertilisation and larval release, larvae are held within these collection trays until approximately 6 days post larval release, they are then sorted by size on mesh sieves and manually transferred into upwelling conical tanks to mature further before settlement. Tanks situated outside of the hatchery are often used as environment for larvae settlement on the substrate of clutch. Water is supplied directly into the hatchery from Langstone harbour is treated through a sand filter, 100, 50, 10 and 1µm cartridge filters and a UV light before being stored in header tanks before use in larval culture. This hatchery also produces microalgae via the neutralisation of sea water on site in maximum culture volumes of 10 L in order to sustain larval cultures. A summary of this process can be seen in Figure 9.

The production of Crassostrea gigas at Morecambe Bay Oysters

This chapter also covers a strip spawning and larval rearing cycle of *C. gigas* conducted at Morecambe Bay Oyster (Seasalter Walney Ltd) shellfish hatchery located remotely in the North West of England (Figure 4). This hatchery operates purely for commercial purposes and alongside Guernsey Sea Farms supplies most of the UK's oyster farms with seedstock (Regan et al, 2021). This hatchery is notably larger than the research hatchery at Portsmouth University.

This hatchery is built amongst a series of artificial ponds, the largest of which holds seawater sourced directly from Morecambe Bay. Other ponds are used to produce algae for nursery systems, for which they are fertilised regularly to boost algal output. Finally, some of the

ponds hold flupsy (floating upwelling system) rafts on which oysters grow rapidly with a high level of feed input and water turnover. Slightly different to Portsmouth, the seawater that enters the hatchery system is this treated through pasteurisation, cartridge filters, and UV exposure before being used within the hatchery. This seawater is then held within holding tanks which directly feed to algae, oyster broodstock, larvae, or early spat. Following a strip spawning, subsequent oyster larvae are held in 200 L upwelling tanks where they are drip fed cultures of live microalgae through a syringe. Samples of larvae are taken daily in order to check larval health and growth. At approximately 25 days post fertilisation, at the pediveliger stage, larvae are induced into metamorphosis via treatment with epinephrine. The resulting spat are matured either within the hatchery in shallow trays, or in outdoor tanks or rafts.

This hatchery also has capacity to produce high volumes of live microalgae, seeded from axenic starter cultures from CCAP (Culture collection of algae and protozoa), which are upscaled with sea water and algal media (essential nutrients) to volumes of 400 L which are maintained with a constant air supply. A summary of this process can be seen in Figures 15 and 16.

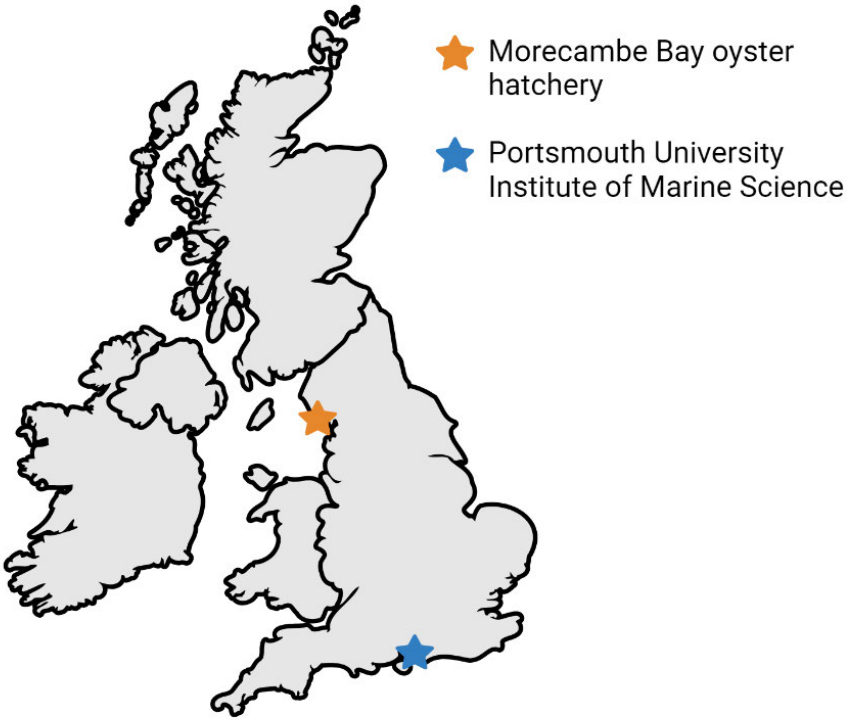


Figure 4: A map of the UK showing the locations of Portsmouth Universities Institute of Marine Science (*Ostrea edulis* hatchery) and Morecambe Bay Oysters (primarily a *Crassostrea gigas* hatchery). Created in BioRender.com.

Chapter aims:

There are very few studies of the microbiome within industrial oyster hatcheries through production runs. These environments do, however, provide an important opportunity to provide data which is directly relevant to the improvement of oyster aquaculture. This chapter, therefore, investigates this knowledge gap by reporting microbial analysis from a broad range of sample types taken throughout oyster spawning events at two different UK hatcheries (Portsmouth University research facility and Morecambe Bay Oysters commercial hatchery). Conducting research in these working, industrial, environments presents both strengths and limitations for this chapter (to be later discussed), and allows for comparisons with similar aims from previous studies (Arfken et al, 2021), which were also conducted in highly controlled aquaculture systems.

This chapter addresses the following aims:

1. To identify core larval taxa and how these change throughout the rearing process.

This will be achieved through identification of bacterial OTUs present in 100% of larval samples. Understanding core bacterial taxa within healthy larvae, and their changes over time is an important prerequisite in order to inform microbial manipulation strategies and maintain optimal microbial communities. This is ultimately for the benefit of larval health and hatchery production. It is hypothesised that certain bacterial taxa will become more relatively abundant in the later stages of larval development, as a possible consequence of fewer OTUs being exchanged with the immediate environment. This hypothesis is informed by previous studies in experimental hatcheries, using *C. gigas* and Crassostrea sikamea (Kumanoto oyster) (Asmani et al, 2016; Dai et al, 2022). These studies report taxa belonging to Flavobacteria (Asmani et al, 2016), and Rhodobacteraceae (Dai et al, 2022), to become more relatively abundant as larval development progressed. This trend over time may be evidence of a shift towards a more selective microbiome as larvae age, where the most beneficial taxa become more established.

2. Compare bacterial profiles of larvae to those of their hatchery environment.

This aim incorporates analysis of larval samples as well as larval culture water, hatchery water, algae and biofilm. This analysis helps us to understand how hatchery conditions influence the microbiome of larvae, and identify possible sources of larval OTUs. It is hypothesised that the larval microbiome will be distinct to that of their surrounding water. This hypothesis is informed by previous studies which report the microbiome of *C. virginica* larvae (Arfken et al, 2021), and *O. lurida* gut to be significantly different to their seawater (Kunselman et al, 2022). In the commercial environment this relationship was driven by higher relative abundances of Flavobacteriia in sea water and Gammaproteobacteria in larvae (Arfken et al, 2021). This differentiation may be evidence of alternative sources of the larval microbiome (other than sea water), or specialisation of the larval microbiome (Unzueta-Martínez et al, 2022). This aim will improve understanding of microbial transfer and establishment; another important prerequisite needed in order to improve hatchery practices and develop methods microbiome manipulation.

3. Investigate how hatchery processes specific to these locations including water filtration and algal cultivation methods influence microbial communities.

Bacterial compositions of samples taken throughout the water filtration and algal cultivation processes will be analysed. This will also aid the understanding of how hatchery processes influence the microbiome of larvae and provide practical information to the hatcheries on the efficiency of these practices. Relative abundance profiles of the *Vibrio* genera across all sample types will also aid in identifying possible sources of microbial contamination to larval cultures. It is hypothesised that specific steps in the water filtration process will reduce microbial diversity and relative abundance of potentially harmful bacteria. This is based on the rationale that water filtration is designed to lessen the viable bacterial load of water used in larval culture. This aim will therefore provide information about the efficiency of these practices.

Methods:

Sample collection and processing methods were kept as consistent between the two hatcheries as possible to reduce variability in results from methodological differences. However, these are significantly different hatcheries and species, and as such there is some variation. As such, the sampling processes detailed below are for each hatchery individually.

Sample collection from Portsmouth IMS:

Samples of larvae, algae, pallial cavity water, larval culture water (the water in which larvae are being cultured, but without the larvae) and hatchery water (water from various hatchery locations and stages throughout the filtration process, except the larval tanks themselves) were collected from the Portsmouth IMS. The sample collection and processing techniques for each of these sample types are outlined below. After processing, each sample was immediately frozen at -20°C in a portable freezer, which was also used for sample storage and transportation back to the Roslin Institute, UK.

Pallial cavity water samples:

Samples of water from within the pallial cavities of four different broodstock oysters were also sampled. To collect this, shells were opened by relaxing oysters in a solution of seawater and Magnesium Chloride (Figure 5), which allowed access to the pallial cavity where water could be extracted with a micropipette. Each sample consisted of 1 mL of pallial cavity water placed into a 1.5 mL Eppendorf.

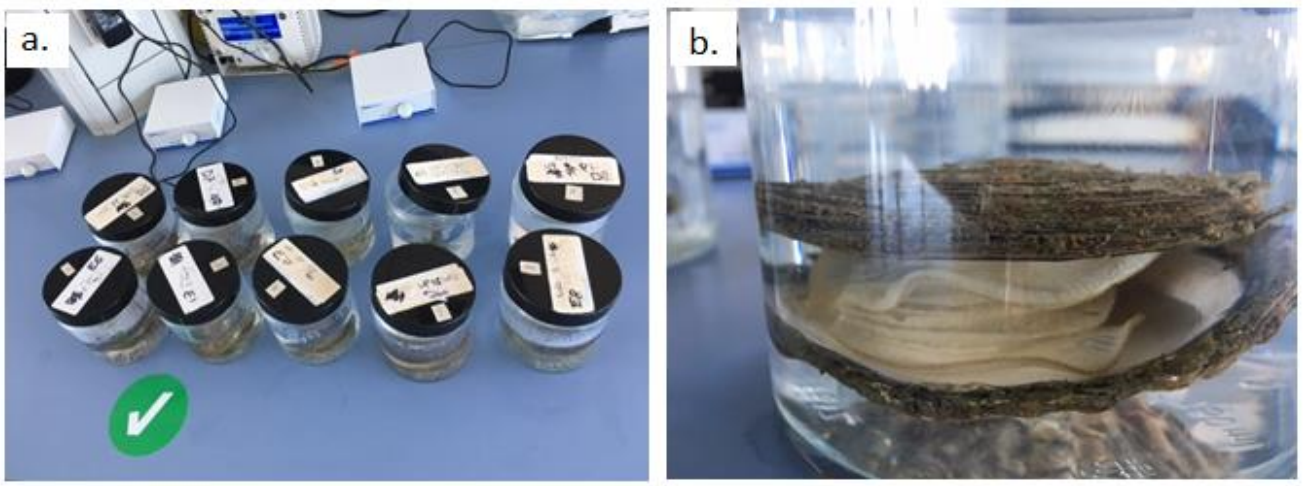


Figure 5: Photographs taken during the collection of pallial cavity water samples from *O. edulis.* **(a)** Individual broodstock oysters were places in individual plastic containers for treatment with Magnesium Chloride. **(b)** Close up image of an *O. edulis* individual once treated with Magnesium Chloride.

Hatchery water samples:

The term 'hatchery water samples' will be used to describe water samples collected from any location around the hatchery (excluding larval cultures). This incorporates water samples collected at various stages of the water filtration process. Hatchery water samples were collected using a sterile 500 mL Erlenmeyer flask which was filled with 250 mLs of the water sample. Samples were then filtered through a 0.22 µm cellulose nitrate membrane filter using a vacuum pump (Figure 6). The remaining filter paper was then carefully rolled using sterile tweezers and folded into a 1.5 mL Eppendorf tube. Each filter paper was then covered with ATL buffer (Qiagen, Hilden, Germany). The filtration apparatus and tweezers were sterilised in-between samples with a 70% ethanol solution.

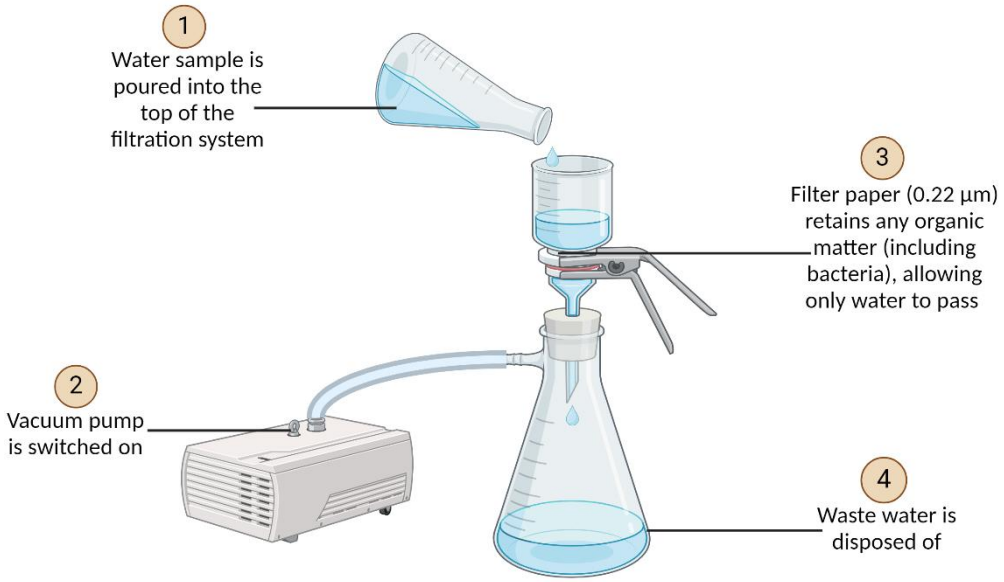


Figure 6: Schematic showing the water filtration process used to process hatchery water samples and larval culture water samples. **1.** The water sample is poured into the top of the water filtration system. **2.** The vacuum pump is switched on, causing the water to be pulled through the filter paper. **3.** The filter paper retains organic matter (including bacteria) within the water sample. **4.** Waste water passes through the filter paper and is disposed of. Created in BioRender.com

Larval culture water samples:

The term 'larval culture water' will be used to describe water samples collected from larval upwelling tank or collection trays (that do not contain larvae). These samples were collected during water changes of the tank or tray (a practice required to maintain optimal conditions for larval health). During a water change, upwelling tanks were drained, and larvae retained on an appropriately sized mesh sieve. As water passed through sieve, 250 mLs were collected in a sterile 500 mL Erlenmeyer flask (Figure 7). This water sample was then filtered as previously described (Figure 6).

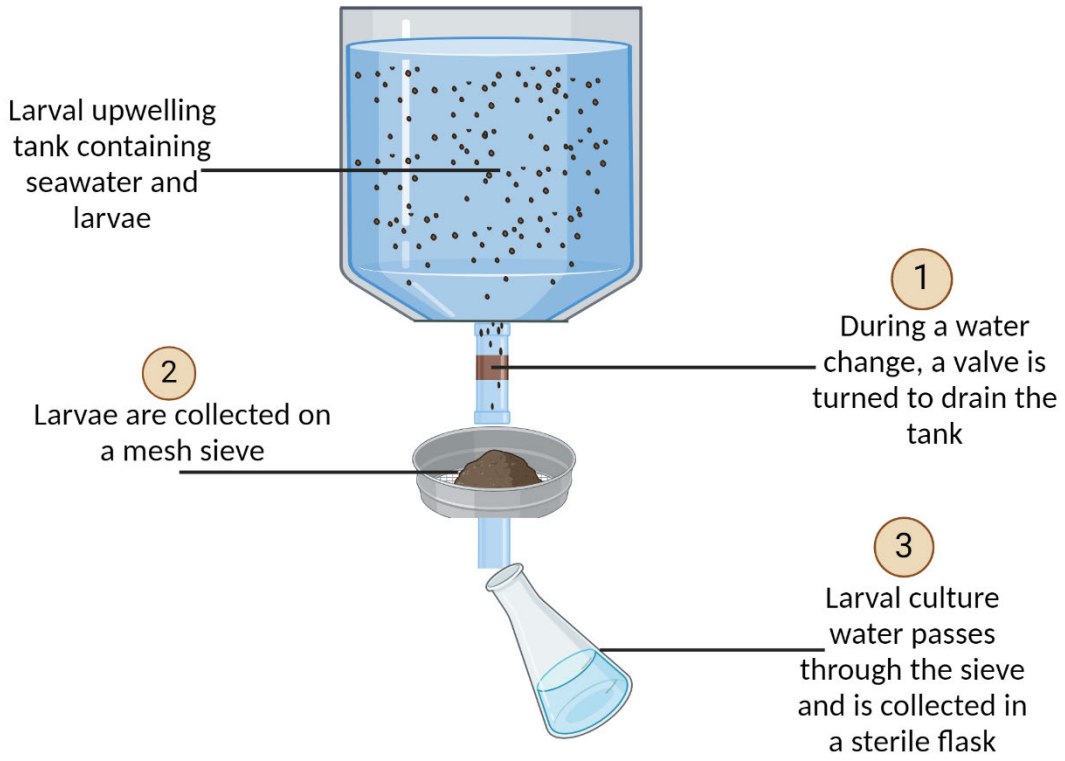


Figure 7: Schematic showing the water change process of a larval upwelling tank which provided opportunity to collected larval culture water samples. **1.** A valve is turned to drain the upwelling tank. **2.** Larvae are collected on an appropriately sized mesh. **3.** Larval culture water passes through the sieve and is collected in an Erlenmeyer flask for further processing. The size of larvae within this figure have been exaggerated to ensure clarity of the water change process. Created in BioRender.com

Larval samples:

Larval samples were collected from a mid-depth in larval collection trays or upwelling tanks using sterile Pasteur pipettes, where 1 mL of larvae were placed into 1.5 mL Eppendorf tubes (Figure 8a).

Algal samples:

Samples of algae and neutralised seawater were collected directly from the culture bags (Figure 8b). Sterile Pasteur pipettes were used to fill 1.5 mL sterile Eppendorf tubes with 1 mL of the sample.

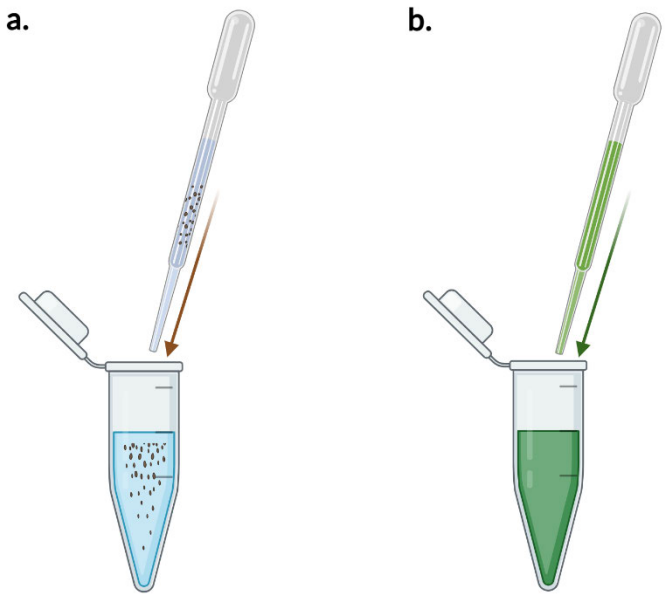


Figure 8: Schematic showing collection of **(a)** larval and **(b)** algal samples. Created in BioRender.com.

A schematic of the sample collection process and layout of the hatchery environment is shown below (Figure 9), alongside photographs of key hatchery locations (Figure 10). Sample collection was carried out between 03/08/2021 – 13/08/2021 after larval release was observed from broodstock oysters in one tank on 03/08/2021 and in another tank on 04/08/2021. This sample collection therefore follows larvae from these two separate sets of broodstock until they were merged equally (and split equally) into three larger larval bins on 09/08/2021 to accommodate further larval growth. These tanks are referred to as upwellings

one, two and three. Larvae from these bins were merged equally again, (and split equally) across two different tanks on 11/08/2021. These tanks are referred to as upwellings four and five (Figure 11).

A total of 74 samples were selected from this hatchery for 16S rRNA sequencing. A summary of these sample types and their locations of collection within the hatchery are summarised in Table 1 below.

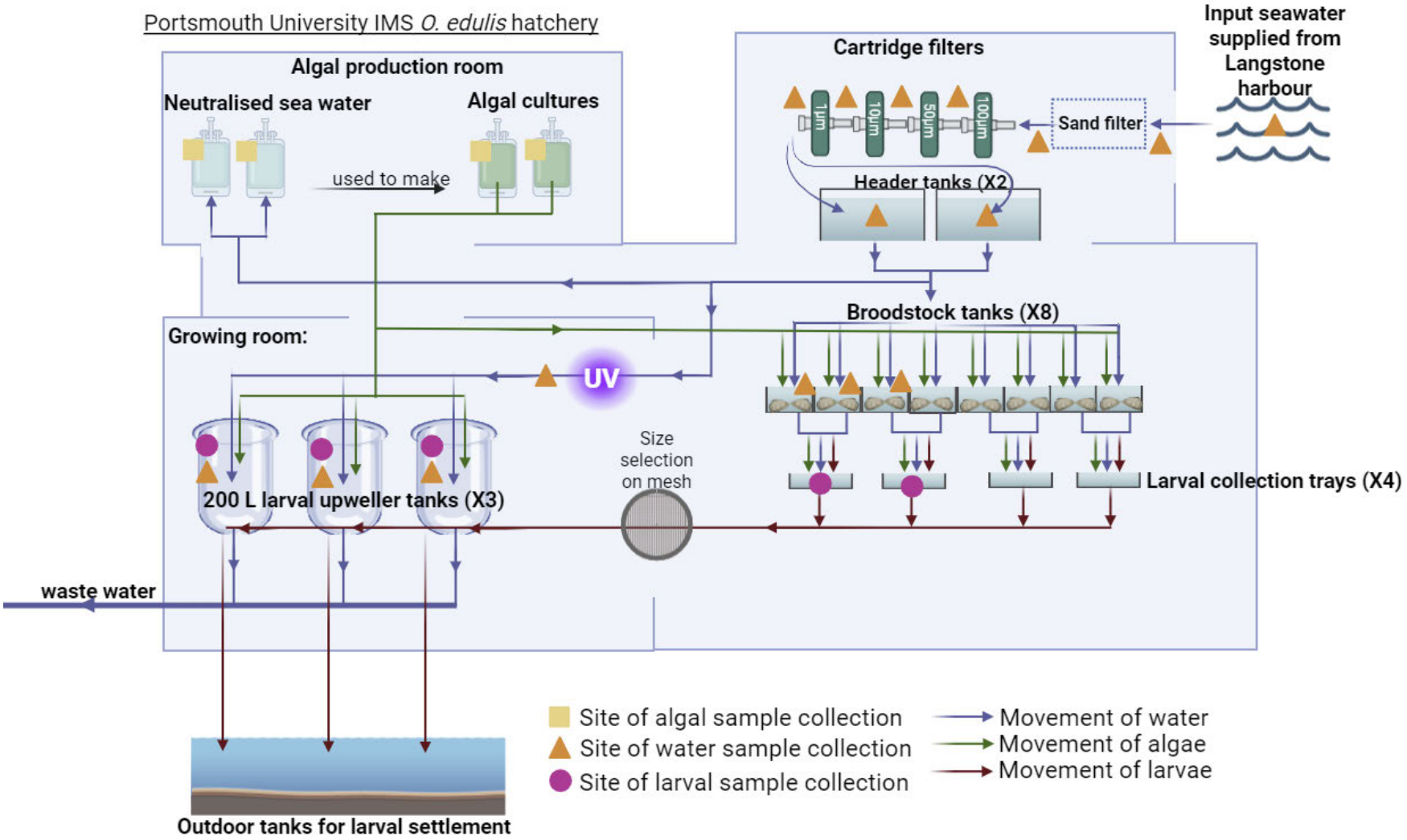


Figure 9: Schematic of the *Ostrea edulis* hatchery at Portsmouth University's IMS. Schematic shows the basic processes of water filtration and movement of larvae and algae throughout the hatchery. Sample collection sites throughout the hatchery environment are also indicated with square, triangle and circle symbols to indicate algal, water and larval sample types retrospectively. Created in BioRender.com

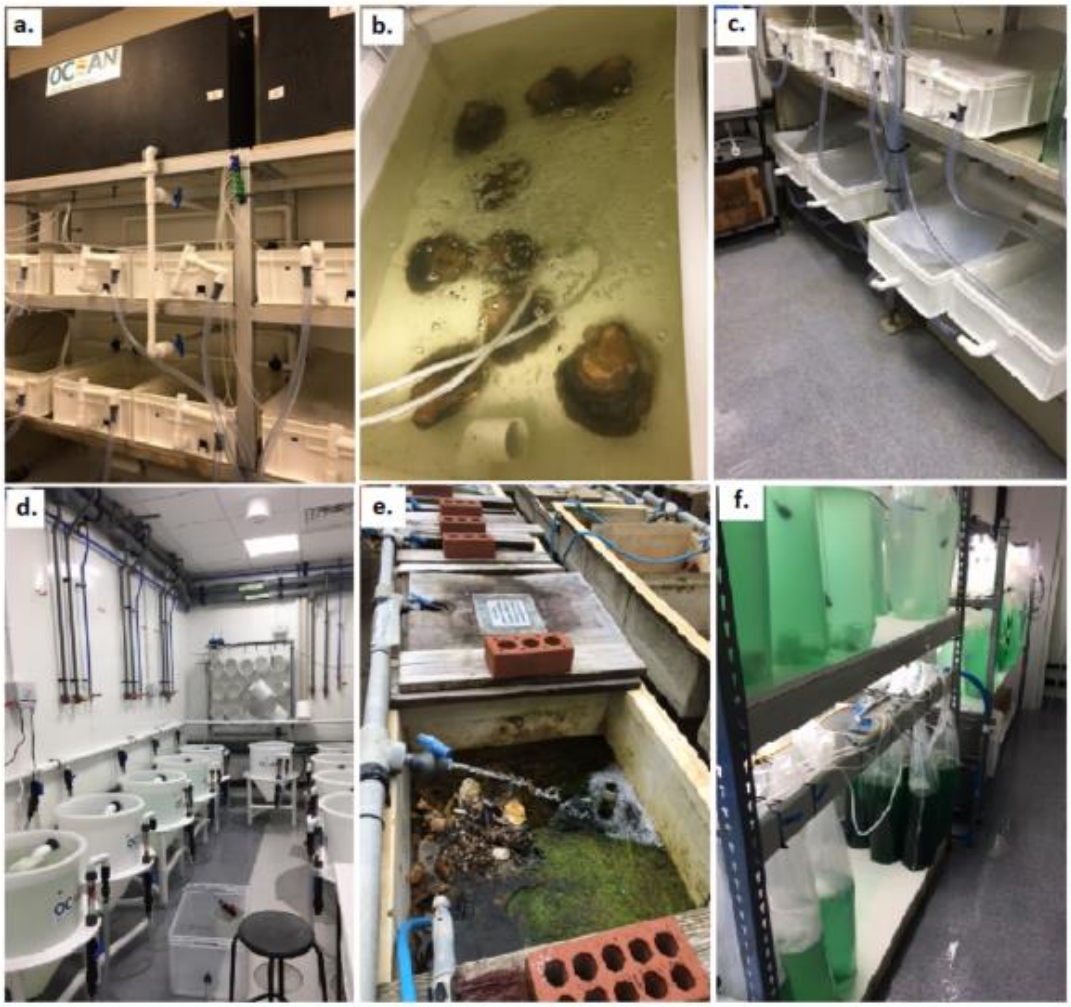


Figure 10: Photographs of key sampling locations within the Portsmouth University *O. edulis* hatchery: **(a)** Seawater header tanks supplying eight oyster broodstock tanks which sit below. **(b)** Broodstock oysters. **(c)** Larval collection trays situated below broodstock tanks. **(d)** Upweller tanks used for rearing larvae. **(e)** Outdoor tanks used for the settlement of larvae on oyster shell clutch. **(f)** Algal production used to feed larval cultures.

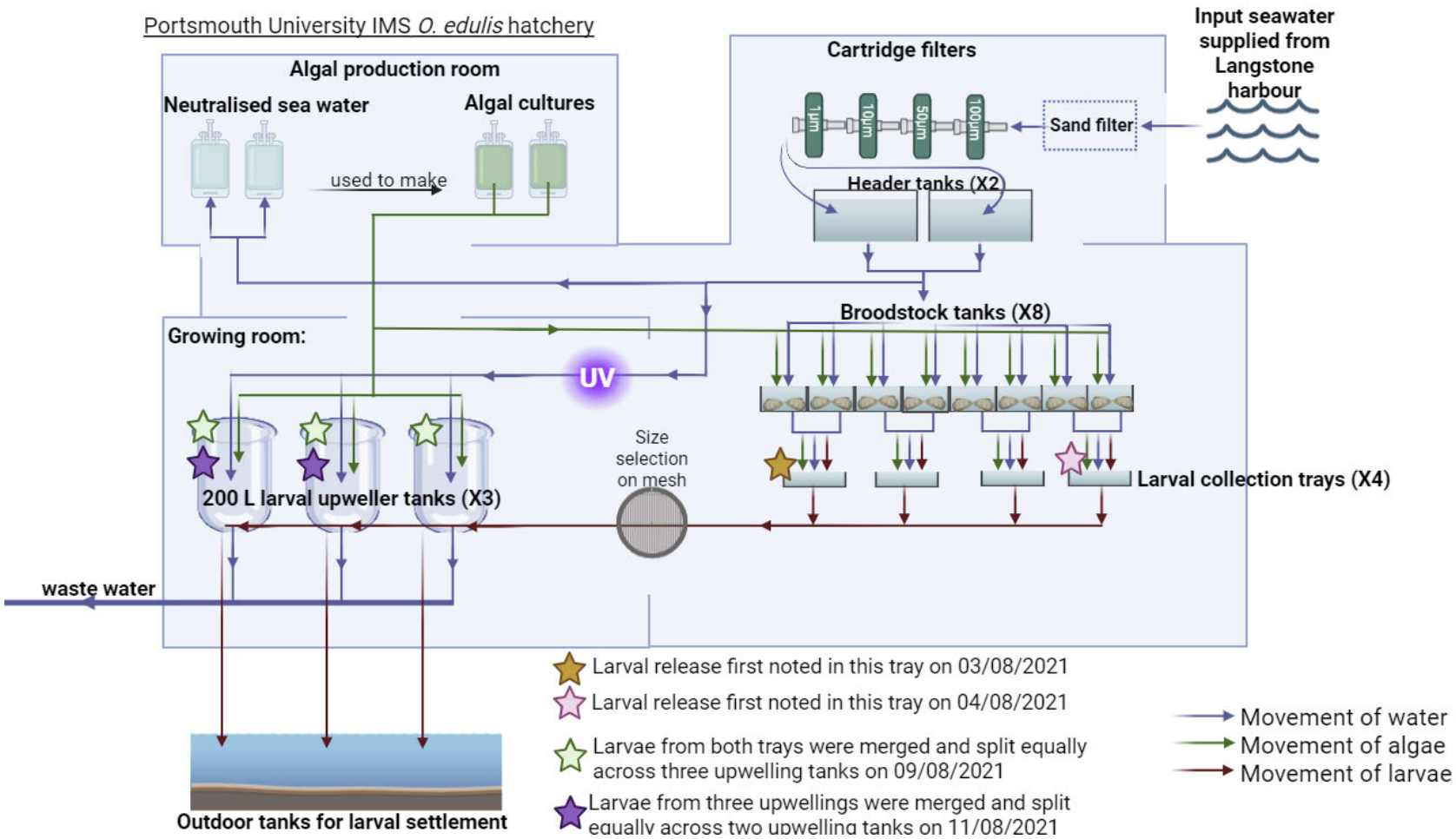


Figure 11: Schematic showing the location and date of larval releases recorded at Portsmouth University's IMS. The first larval release was recorded on 03/08/2021. Larval release was recorded in a separate collection tray on 04/08/2021. Larvae were sampled from these separate strays before being equally merged and split across three upwelling tanks on 09/08/2021. On 11/08/2021 larvae from the three upwelling tanks were equally merged and split across two upwelling tanks (referred to as upwellings four and five). Created in BioRender.com

Table 1: A summary table showing the 74 samples collected throughout the *O. edulis* spawning event at Portsmouth University ISM which were selected for 16S sequencing. Symbols of: ☑ = collection of a water sample, ☑ = collection of a larval sample ☑ = collection of an algal sample and ☑ = collection of a pallial cavity sample.

	Days post larval release									
Location	0	1	2	3	5	6	7	8	9	10
Larval tray 1	☑	☑	☑	☑						
Larval tray 2		☑	☑	☑						
Broodstock tank 1	☑	☑	☑	☑	☑					☑
Broodstock tank 2	☑	☑	☑	☑	☑					☑
Broodstock tank 3	☑				☑					☑
Larval upweller 1						☑	☑☑	☑☑	☑	☑☑
Larval upweller 2						☑☑	☑☑	☑	☑	☑
Larval upweller 3						☑☑	☑☑			
Harbour inlet	☑				☑					☑
Pre sand filter	☑				☑					☑
Post sand filter										☑
Post 100 µm filter	☑			☑						
Post 50 µm filter	☑	☑		☑						
Post 5 µm filter	☑	☑		☑						
Post 1 µm filter	☑	☑		☑						
Post UV	☑									
Header tank 1	☑				☑					☑
Header tank 2	☑				☑					☑
Algal culture			☑☑☑☑							
Pallial cavity							☑☑☑☑			

Sample collection from Morecambe Bay oyster hatchery:

Samples of gametes, larvae, algae, biofilm, larval culture water (containing no larvae) and hatchery water (water from various hatchery locations and stages throughout the filtration process) were collected following a strip spawning event Morecambe Bay oyster hatchery. The sample collection and processing techniques for each of these sample types are outlines below. After processing, each sample was immediately frozen at -20°C in a portable freezer, which was also used for sample storage and transportation back to the Roslin Institute, UK.

Biofilm samples:

Biofilm samples were collected by swabbing the surface of interest with a sterile cotton swab. The stem of the swab was then snapped in order to fit the head of the swab in a 1.5 mL Eppendorf tube (Figure 12). Approximately 1 mL of ATL buffer was then added to the Eppendorf tube.

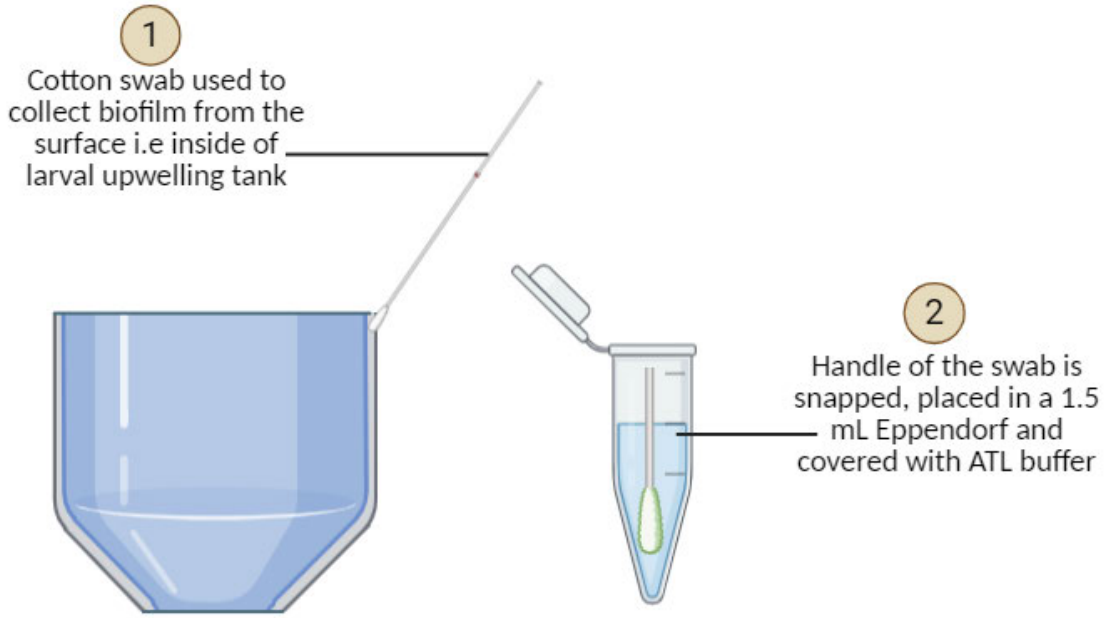


Figure 12: Schematic showing the sample process of biofilm samples. Created in BioRender.com.

Hatchery water samples:

Hatchery water samples were collected using a sterile 500 mL Erlenmeyer flask which was filled with 250 mLs of the water sample. Samples were then filtered through a 0.22 µm cellulose nitrate membrane filter using a vacuum pump (Figure 6). The remaining filter paper was then carefully rolled using sterile tweezers and folded into a 1.5 mL Eppendorf tube. Each filter paper was then covered with ATL buffer. The filtration apparatus and tweezers were sterilised in-between samples with a 70% ethanol solution.

Larval culture water samples:

The term 'larval culture water' will be used to describe water samples collected from larval upwelling tank or collection trays (that do not contain larvae). These samples were collected by filling a sterile 500 mL Erlenmeyer flask with larvae and their surrounding water from the mid-depth of a larval upwelling tank. The contents of the flask was then passed through an appropriately sized mesh sieve to retain the larvae. The water passing through the sieve (containing no larvae) was then collected in another sterile 500 mL Erlenmeyer flask (Figure 13). This water sample was then filtered as previously described (Figure 6).

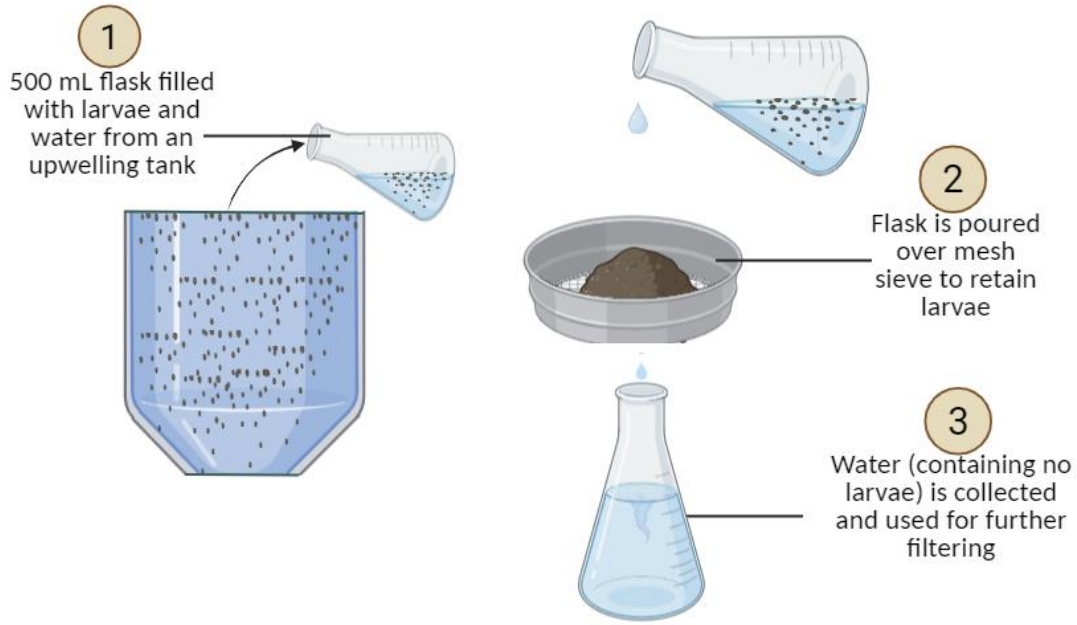


Figure 13: Schematic showing the collection process of larval culture water samples at Morecambe Bay oyster hatchery. Created in BioRender.com.

Gamete samples:

Broodstock oysters were shucked and the flesh of the animal removed from its shell. The oyster flesh was then rinsed in a bleach solution (a common practice of this hatchery to reduce the risk of contamination by microorganisms), and then again with filtered sea water to remove bleach residue A sterile blade was then used to make an incision on the gonad of the oyster (Figure 14). Gamete cells were then washed into a glass beaker and subsamples taken for identification under a light microscope for the presence of egg or sperm cells. Gamete samples were collected by using a sterile Pasteur pipette to add 1 mL of the samples into a 1.5 mL Eppendorf.



Figure 14: Photograph of an opened oyster, with red arrow highlighting the gonad (tissue in which eggs and or sperm cell production and storage occurs).

Larval samples:

Larval samples were collected from mid-depth in upwelling tanks using sterile Pasteur pipettes, with 1 mL of larvae placed into 1.5 mL Eppendorf tubes (Figure 8a).

Algal samples:

Samples of *Tisochrysis lutea (T. iso)* and *Pavlova* were collected directly from starter cultures or the from the fully established 400 L algal cultures (Figure 8b). Sterile Pasteur pipettes were used to fill 1.5 mL sterile Eppendorf tubes with 1 mL of the sample. Algal production at Morecambe Bay involves the upscaling of algal cultures over time. This involves the purchase

of an axenic starter culture which is grown in progressively large volumes overtime (to a maximum of 400 L). Samples were taken from both axenic starter cultures and 400 L culture bags.

Schematic diagrams of the sample collection process (including both outside and inside of the hatchery) are shown below (Figures 15 and 16). Photographs taken throughout the sampling process are also shown (Figure 17). A strip spawning was conducted on 16/05/2022, and the subsequent larvae were treated with epinephrine to induce settlement on 10/06/2022. Samples collection as therefore conducted between 16/05/2022 and 11/06/2022, to include the development of larvae from spawning to settlement. Larvae from the same broodstock were split equally and reared across three separate upwelling tanks. The largest larvae from these tanks were then selected for on mesh sieves and evenly split into two upwelling tanks on 25/05/2022 (referred to as upwellings four and five); (Figure 18). A total of 99 samples were selected from 16S rRNA sequencing and are summarised below (Table 2).

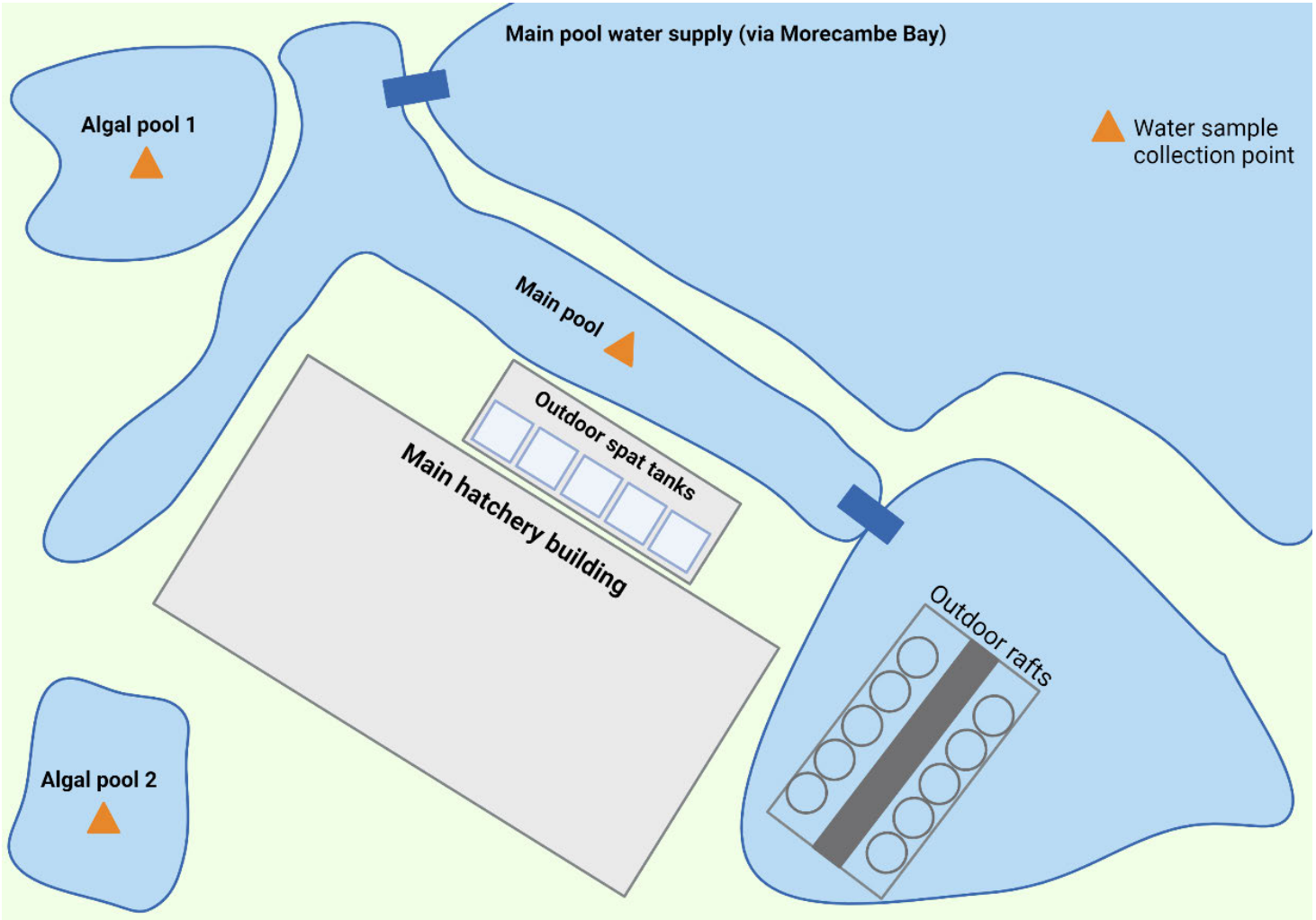


Figure 15: Schematic of the Morecambe Bay oyster hatchery from above, including water sources which supply the hatchery, pools used as a method of algal production and location of outdoor rafts used for maturing oysters outside. Created in BioRender.com.

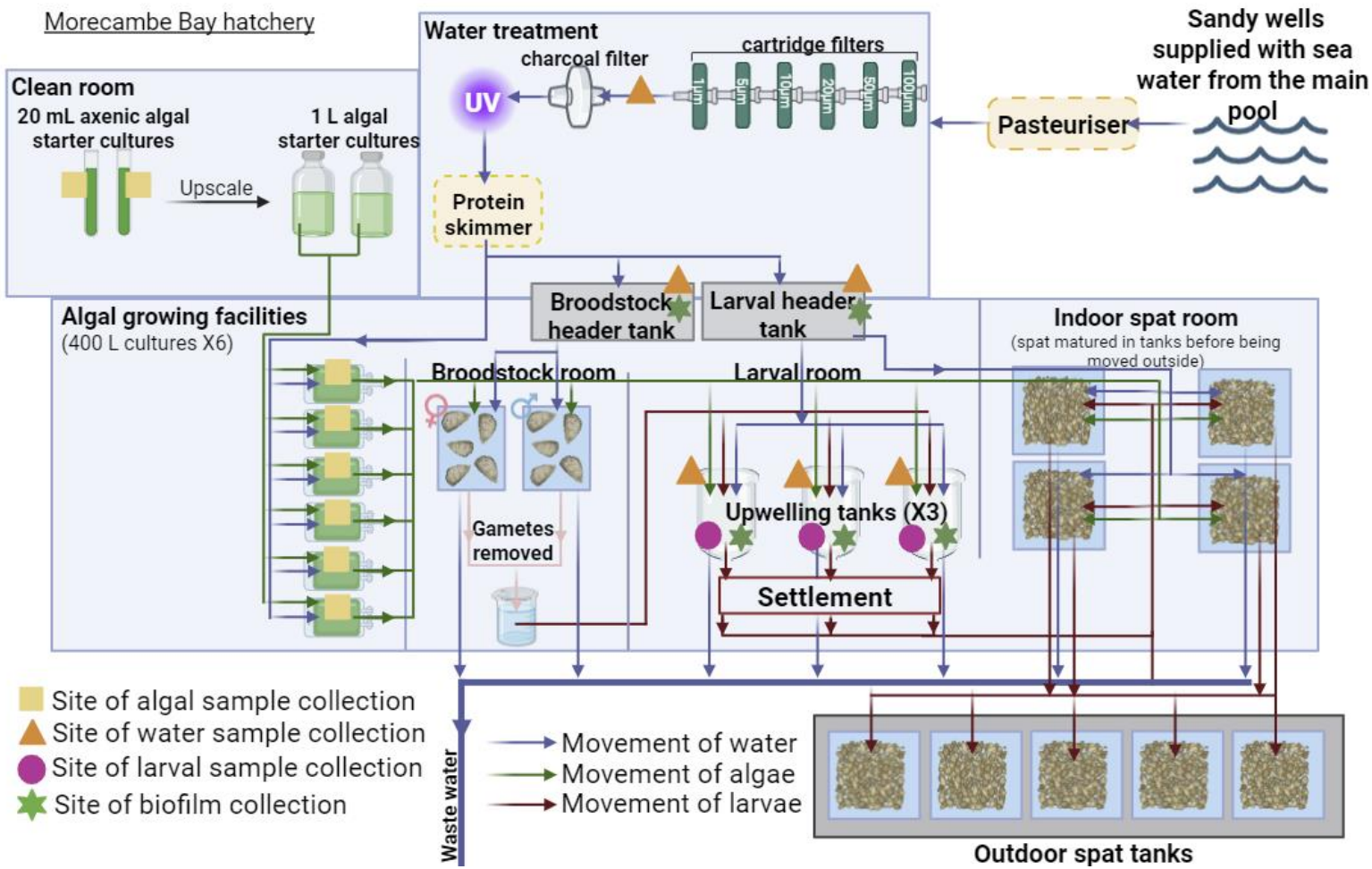


Figure 16: Schematic showing the inside of Morecambe Bay hatchery highlighting water filtration methods, production of algae and development of larvae throughout a hatchery cycle following strip spawning and external fertilisation. The movement of water, algae and larvae throughout different systems of the hatchery are indicated with arrows and sample collection sites and shown with square, triangle, circle and star symbols for algal, water, larval and biofilm sample types. Created in BioRender.com.



Figure 17: Photographs from Morecambe Bay hatchery showing **(a)** The main pool which holds the hatchery's sea water supply from Morecambe Bay **(b)** Header tanks supplying treated seawater to larvae and broodstock. **(c)** Two 200 L upwelling tanks of larval cultures. **(d)** A view of the larval culture room. **(e)** A 200 L larval upwelling tank supplied with a water heater. **(f)** Cultures of live microalgae with a maximum volume of 400 L. **(g)** Cultures of live microalgae with a maximum volume of 400 L.

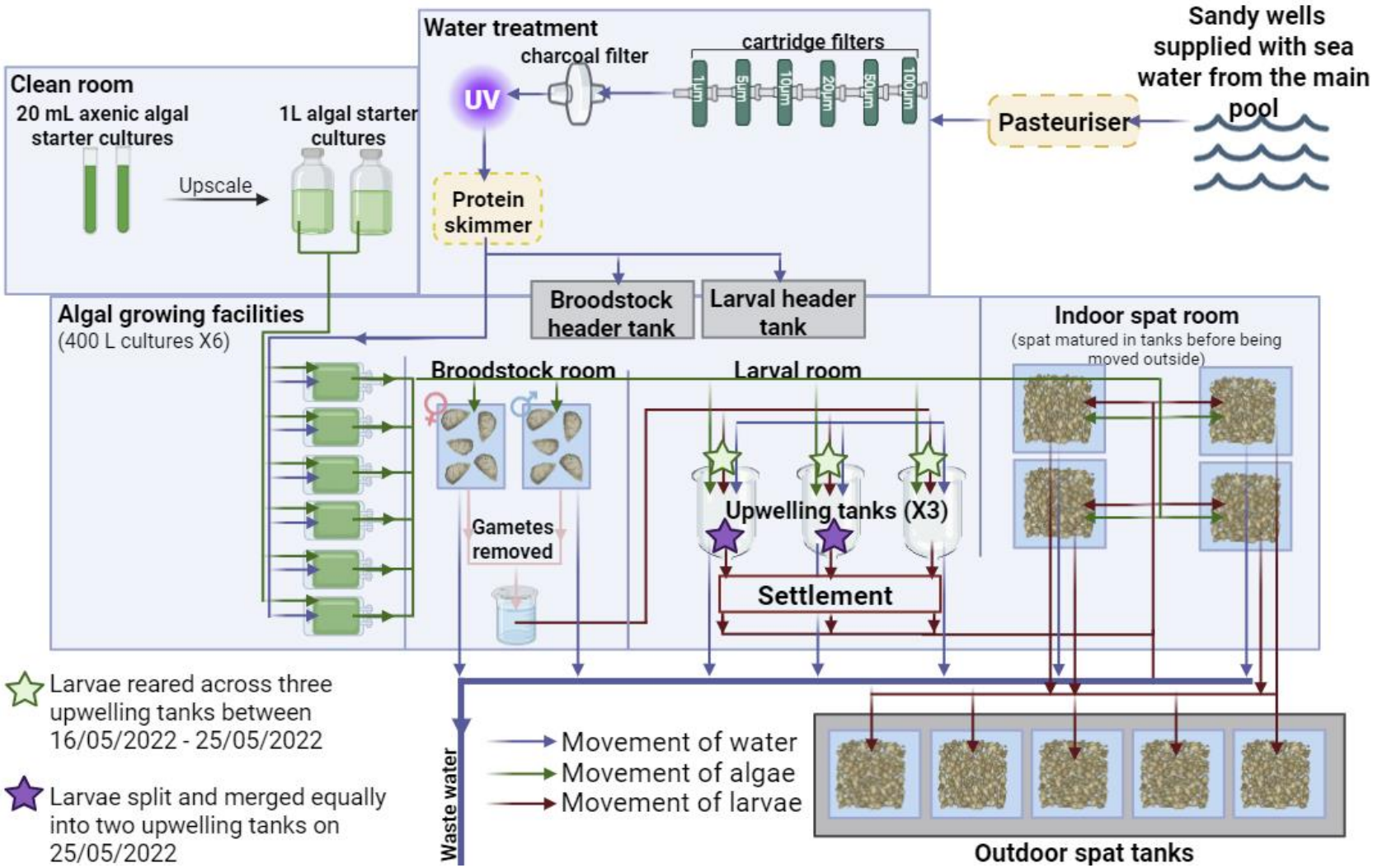


Figure 18: Schematic showing the merging of larvae in the Morecambe Bay hatchery. Larvae from the strip spawning were reared across three upwelling tanks (upwelling tanks one, two and three). On 25/05/2022, the contents of these upwelling tanks were equally merged and split across two upwelling tanks (referred to as upwelling tanks four and five). Created in BioRender.com.

Table 2: A summary of the 99 samples selected from 16S rRNA sequencing from Morecambe Bay. The location and time of each sample collection is given, where the strip spawning was carried out at 0 days post fertilisation. Symbols of '☑' are used to indicate collection of one sample which are coloured according to sample type: green = algal sample, red = gamete sample, blue = water sample, yellow = biofilm sample and orange = larval sample.

	Days post fertilisation														
Location	0	1	2	3	5	7	8	9	11	14	17	21	24	25	26
Algal starter culture	☑☑														
Broodstock oysters	☑☑☑☑☑☑														
Larval header tank	☑	☑	☑	☑	☑☑	☑		☑☑	☑	☑	☑	☑	☑	☑	
Larval upweller 1	☑	☑☑		☑	☑☑	☑		☑☑	☑☑	☑☑	☑☑	☑☑	☑☑☑		☑☑
Larval upweller 2	☑	☑☑			☑☑	☑		☑☑	☑☑	☑☑	☑☑	☑☑	☑☑		☑
Larval upweller 3	☑	☑☑			☑☑	☑									
Main pool		☑				☑								☑	
Inside hatchery (pre UV)		☑	☑			☑								☑	
Algal pool 1		☑				☑								☑	
Algal pool 2		☑				☑								☑	
Broodstock header tank		☑	☑		☑	☑		☑						☑☑	
40L algal cultures							☑☑☑☑☑☑						☑☑☑☑		

Sample preparation:

After defrosting at room temperature for 20 minutes, the entire contents of each Eppendorf (including filter papers or swabs) were transferred into 1 mL Matrix A tubes from MP Biomedicals. Each sample was then placed into an MP Biomedicals FastPrep machine for two rounds of bead-beating, performed at a speed of 5.0 m/s for 40 seconds. After checking that each sample appeared homogenous, 3 µL of Metapolyzyme (Sigma Aldrich #MAC4L – 5MG) was added and briefly vortexed. Samples were then incubated at 37°C for two hours. Each sample then had 20 µL of Proteinase K added, was briefly vortexed and incubated at 56°C overnight. Following this digestion, samples were vortexed, and centrifuged at 13,000 x g for one minute. A minimum of 900 µLs of supernatant was removed and transferred into a sterile 1.5 mL Eppendorf. This centrifugation step was then repeated and 600 µls of the supernatant was transferred into a sterile 1.5 mL Eppendorf and was used as the lysate for DNA extraction.

Molecular Biology:

DNA extraction was conducted using the Maxwell RSC Instrument with the PrueFood GMO and Authentication kit. This process was carried out in accordance with the manufacturer's instructions, where magnetic bead-based technology allows for DNA extraction.

This DNA was then used as a template in a PCR reaction with 16S universal primers (F= AGRGTTYGATYMTGGCTCAG R= VGYTACCTTGTTACGACTT) and Oxford Nanopore library preparation as previously described in Chapter two. After barcode ligation with the Native Barcoding Expansion Kit (EXP-NBD196) from ONT, the LSK-112 library preparation protocol from ONT was resumed from the end-prep stage onwards to allow the protocol to be compatible with the MinION R12 flow cell. The pooled libraries were then loaded onto four separate flow cells, with two being used for samples obtained each hatchery location. Sequencing ran for 68 hours and 24 minutes with no live basecalling. At the end of sequencing, a total of 11.93 million reads were obtained from the Morecambe Bay sample set, and 8.13 million reads from the Portsmouth sample set.

Data handling and analysis:

Initial processing of sequence data:

The initial processing of FAST5 files produced from Nanopore sequencing were processed as described previously in chapter two. This resulted in an final OTU table which was used an input for all further downstream analysis in R Studio (v3.6.1). Prior to OTU analysis, low abundance taxa where the maximum proportion of reads per sample was less than 0.0001. Using the Phyloseq package, the data was processed into a Phyloseq object, requiring an OTU, sample metadata and taxonomy table. The 'subset_taxa' function was then used to remove Chloroplast and Mitochondrial OTUs. This Phyloseq object was used for further OTU analysis.

Generation of heatmap figures:

All heatmap figures were produced in R Studio (v3.6.1), using the Phyloseq, Pheatmap and ggplot2 packages. Phyloseq objects were filtered based on sample metadata (as required) using the 'subset_sample' function. Phyloseq objects were converted into a data frame using the 'as.matrix' function and relative abundance counts using the 'transform_sample_counts' function. Data frames were standardised using the 'scale()' function for Z- score normalisation. The pheatmap() function was then used in order to create the heatmap with Euclidean clustering being applied to rows and columns as required.

Alpha diversity analysis:

Alpha diversity analysis was conducted in R Studio (v3.6.1), using the Phyloseq and ggplot2 packages. The 'estimate_richness' function of the Phyloseq package was used to calculate the number of observed features in each sample being analysed as well as their Chao1 indices. These results were visualised with the ggplot2 package as violin plots, with customisations such as average alpha diversity data points of groups being added as required.

Beta diversity analysis:

Non-metric Multidimensional Scaling (NMDS) analysis was conducted in R Studio (v3.6.1), using the Phyloseq and ggplot2 packages. The 'ordinate' function was used to perform NMDS ordination using Bray-Curtis distances. These distance values were then visualised using the ggplot2 package, where samples could be assigned a shape and or colour according to metadata variables of interest.

Where possible, ellipses were incorporated into NMDS analysis. This was done using the 'stat_ellipse' function assuming a t-distribution for calculating the ellipse shape. This produced an ellipses around each group e.g. sample type, indicating the 95% confidence limit for that group. This analysis requires each group of interest to have more than two data points, and so was only conducted for Morecambe Bay samples.

Statistical testing:

Kruskal-Wallis tests were used to test for significant differences between average alpha diversity indices of different groups of interest. This is a non-parametric test (does not assume normal distribution) of the data. The 'kruskal.test' function was used to perform this test using either Observed or Chao1 values in R Studio (v3.6.1).

To test for significant differences between groups shown in NMDS analysis, Permutational Multivariate Analysis of Variance (PERMANOVA) statistical tests were used. This required the use of the 'adonis2' function of the Vegan package in R Studio (v3.6.1).

Relative abundance:

Relative abundance data was presented in bar chart formats using the Phyloseq and ggplo2 packaged in R Studio (v3.6.1). OTU counts were transformed to proportions (relative abundance), using the 'transform_sample_counts' function. The 'tax_glom' function was then used to aggregate to the taxonomic level of interest e.g. Family. The resulting counts were then visualised within ggplot2, using the 'facet_wrap' function to separate according to certain metadata varaibles as required.

Differentially abundant taxa:

This analysis tested for and visualised taxa that significantly differed in abundance between two groups. This was conducted in R Studio (v3.6.1) using packages of: DESeq2, Phyloseq, BiocGenerics, ggplot2, dyplr and tibble. From a Phyloseq object, the metadata variable of interest e.g. 'age group' was set as a factor and the 'relevel' function was used to set the reference level group. The Phyloseq object was then converted to a DESeq2 object before defining and calculating the geometric mean. The DESeq2 analysis was then performed, statistically significant results ($p = < 0.05$) extracted and converted to a data frame. The ggplot2 package was used to plot this data, where taxa were shown on the x axis alongside a log2 fold change on the y axis.

Defining larval developmental stages with data analysis:

Microbial analysis of larval samples collected at Portsmouth were grouped into three categories to include those collected immediately after larval release (maximum of 12 hours), between 13 hours – 6 days post larval release and between 7 – 10 days post larval release.

Microbial analysis of larval samples collected at Morecambe Bay were grouped into four categories to include D-shaped larvae (collected between 12 – 24 hours post fertilisation), early veliger larvae (collected between 3-7 days post fertilisation), late veliger larvae (collected between 9- 24 days post fertilisation) and pediveliger larvae (collected 26 days post fertilisation).

* * *

Results (Portsmouth)

Aim 1: To identify core larval taxa and how these change throughout the rearing process.

A total of 1173 OTUs were identified within the 19 larval samples collected at Portsmouth. In order to identify core bacterial taxa of the larvae, OTUs which were present in each larval sample were selected. From the original 1,173 OTUs identified, 42 were present in each of the larval samples and have therefore been used to describe the core Portsmouth larval microbiome. The relative abundance of these OTUs across each larval sample is shown below, with most taxonomic identifications given at a genus level (Figure 20). OTUs which could not be identified to a genus level are listed instead with their corresponding of bacterial family '*' or order '**'.

Samples cluster loosely according to their larval stage. Larvae sampled between 13 hours and 6 days post larval release had a higher relative abundance of OTUs belonging to *Sulfitobacter*, *Tateyamaria*, *Actibacterium* and *Silicimonas*. Larvae sampled at a later stage of development (between 7 – 10 days post larval release) had a higher relative abundance of *Achromobacter*, *Acinetobacter*, Alcaligenaceae and *Pseudomonadales*. Two larval samples collected immediately after larval release (maximum of 12 hours), are however not clustered closely together. Larvae collected at this time point from tray number one had a higher abundance of OTUs belonging to genera such as *Flavobacteriaceae* and *Ruegeria* compared to larvae collected at the same developmental stage from tray four.

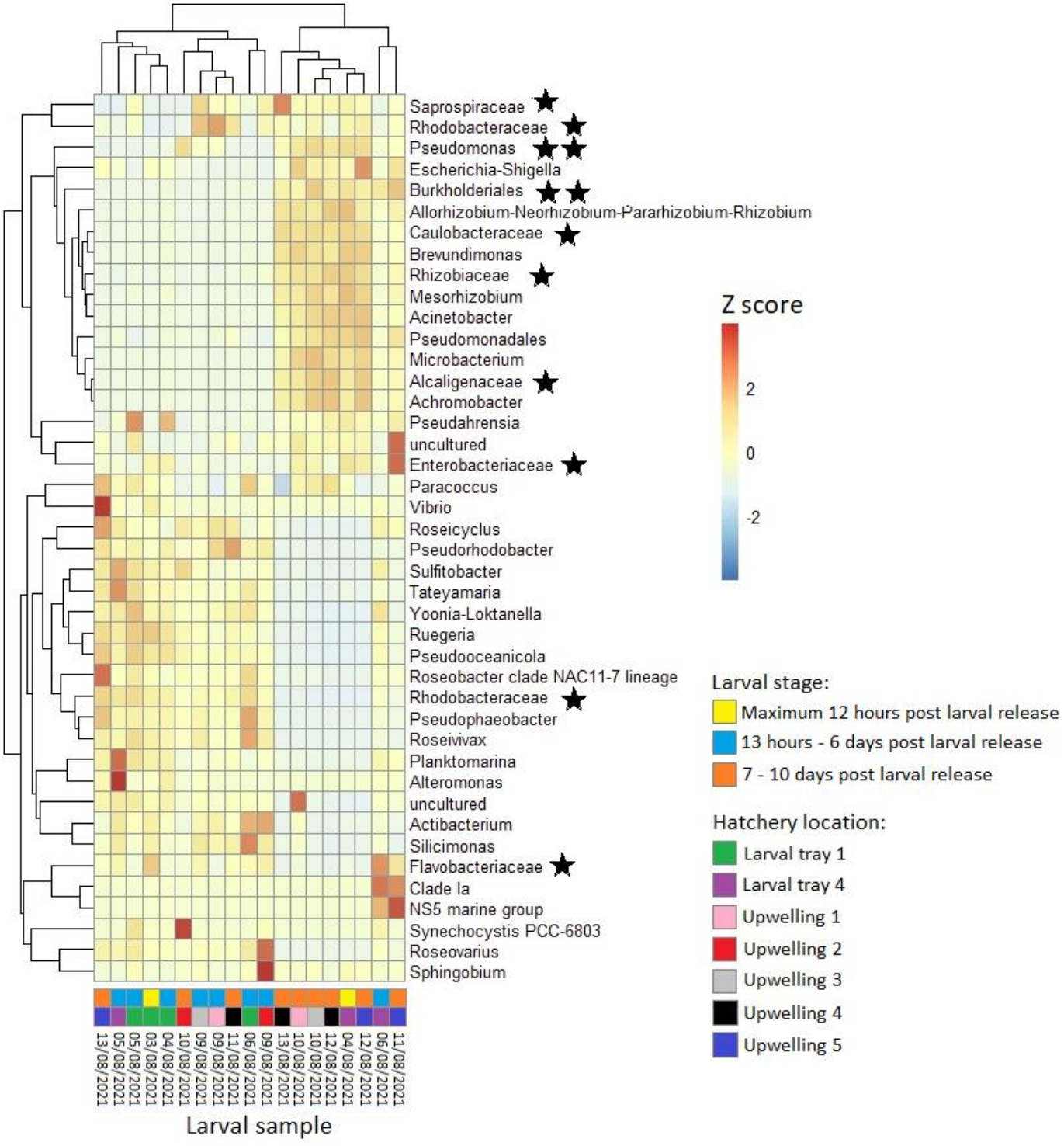

Saprospiraceae
Rhodobacteraceae
Pseudomonas
Escherichia-Shigella
Burkholderiales
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Caulobacteraceae
Brevundimonas
Rhizobiaceae
Mesorhizobium
Acinetobacter
Pseudomonadales
Microbacterium
Alcaligenaceae
Achromobacter
Pseudahrensia
uncultured
Enterobacteriaceae
Paracoccus
Vibrio
Roseicyclus
Pseudorhodobacter
Sulfitobacter
Tateyamaria
Yoonia-Loktanella
Ruegeria
Pseudooceanicola
Roseobacter clade NAC11-7 lineage
Rhodobacteraceae
Pseudophaeobacter
Roseivivax
Planktomarina
Alteromonas
uncultured
Actibacterium
Silicimonas
Flavobacteriaceae
Clade Ia
NS5 marine group
Synechocystis PCC-6803
Roseovarius
Sphingobium
13/08/2021
05/08/2021
05/08/2021
03/08/2021
04/08/2021
10/08/2021
09/08/2021
09/08/2021
11/08/2021
06/08/2021
09/08/2021
13/08/2021
10/08/2021
10/08/2021
12/08/2021
04/08/2021
12/08/2021
06/08/2021
11/08/2021
Larval sample
Z score
2
0
-2
Larval stage:
Maximum 12 hours post larval release
13 hours - 6 days post larval release
7 - 10 days post larval release
Hatchery location:
Larval tray 1
Larval tray 4
Upwelling 1
Upwelling 2
Upwelling 3
Upwelling 4
Upwelling 5

Figure 20: A heatmap of the 42 OTUs present in all 19 larval samples collected from Portsmouth. Each sample is annotated with its corresponding larval stage (sampled at a maximum of 12 hours post larval release, 13 hours – 6 days post larval release and 7 – 10 days post larval release), hatchery location and collection date. The contents of upwelling tanks one two and three were equally merged on 11/08/2021 and distributed equally across two further upwelling tanks. From 11/08/2021 onwards these tanks are therefore referred to as upwellings four and five. Z-score normalisation was performed on relative abundance data and Euclidean clustering was applied to rows and columns. OTUs identified to a family or order level are annotated via '*' and '**' symbols.

* * *

Aim 2: Compare bacterial profiles of larvae to those of their hatchery environment.

NMDS analysis was conducted to assess microbial differences between different sample types (Figure 21). Here, each sample is assigned a colour or shape to denote its origin (algae, hatchery water, larvae, pallial cavity water or larval culture water) and time of collection during larval development. A PERMANOVA statistical test concluded that microbial differences were statistically significant between the different sample types ($p = 0.001$, $r^2 = 0.354$), and larval stages ($p = 0.002$, $r^2 = 0.104$). Larval samples (green), appear most closely grouped with that of larval culture water (pink) and pallial cavity water samples (blue). Larval samples therefore share a microbial composition more similar to these sample types than hatchery water (yellow).

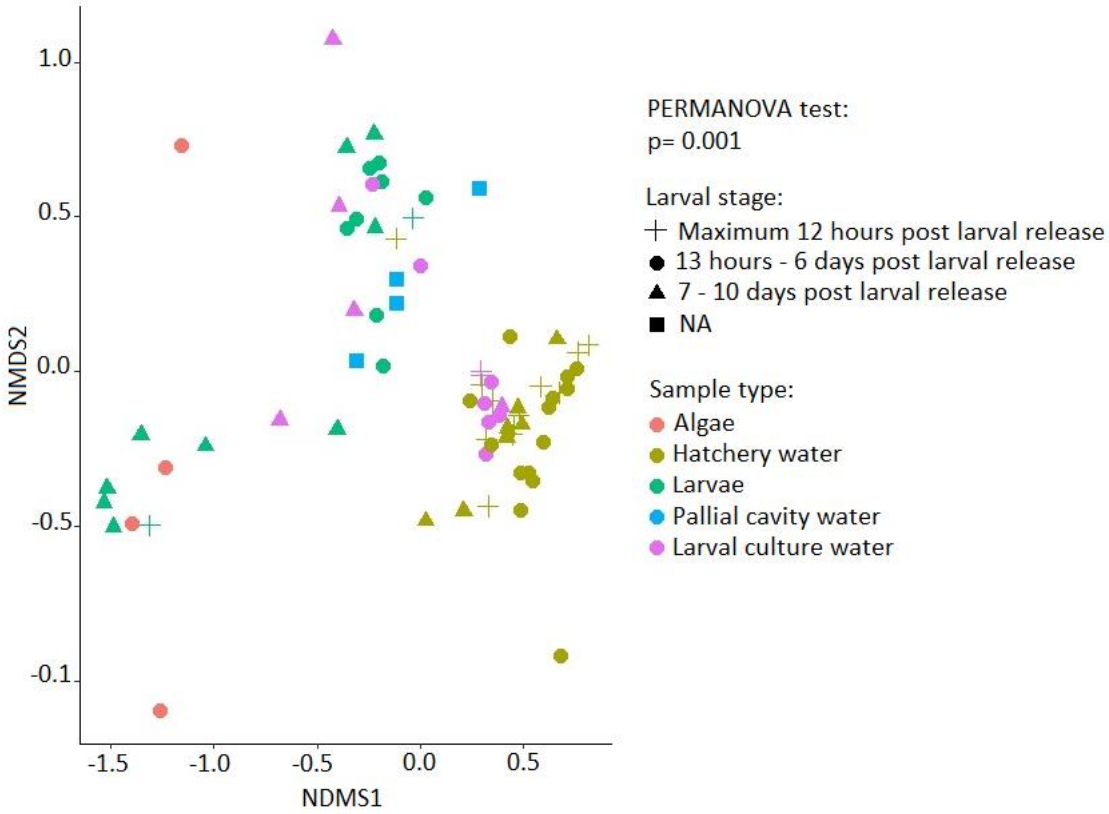


Figure 21: NMDS analysis conducted on all of the 74 samples collected from Portsmouth, where each point represents one sample. Samples are coloured according to sample type covering algal, hatchery water, larval, pallial cavity water and larval culture water. Hatchery water samples cover the range of water samples collected throughout the hatchery environment, and larval culture water samples, contained no larvae. The shape of each sample indicates the life stage of larvae at the time of sample collection.

Alpha diversity indices were calculated between larval samples and their corresponding culture water. Figure 22 shows the average alpha diversity measurements for all larval and larval culture water samples, coloured according to the date on which they were collected (see Figure 22). This shows species richness of both larvae and their culture water to have decreased throughout the spawning. A Kruskal-Wallis test confirmed there to be no statistically significant difference in species richness between these two sample types: Observed (p=0.1932) and Chao1 (p=0.1205).

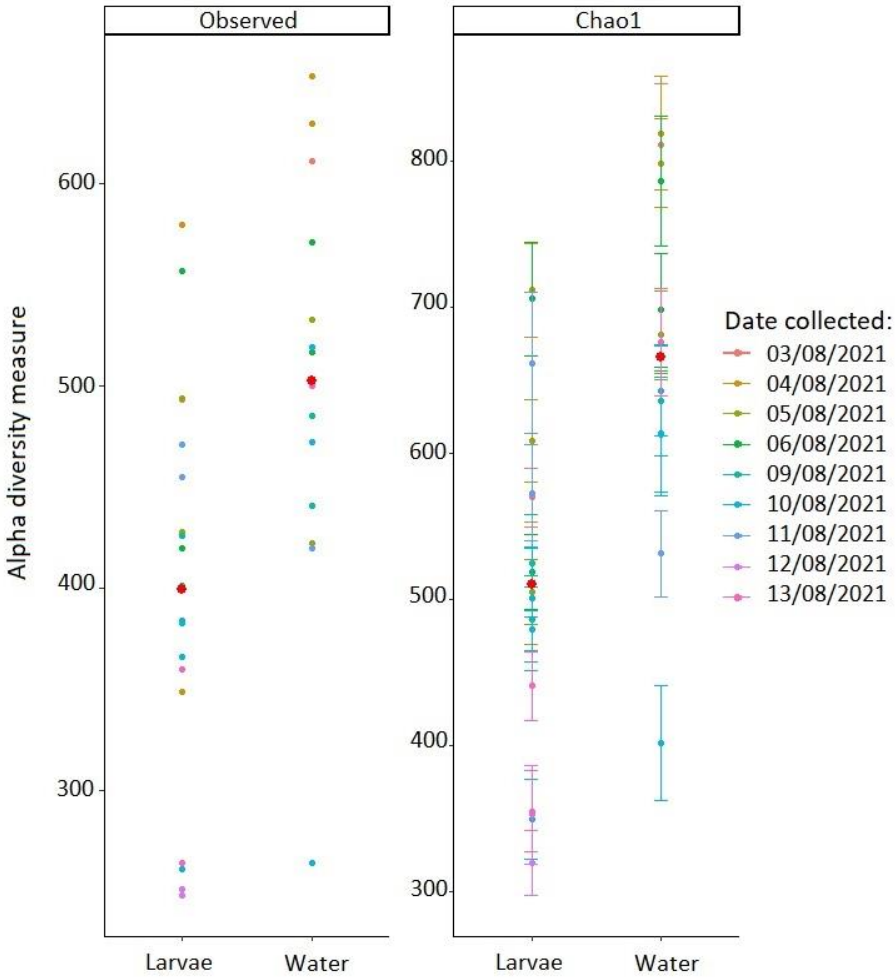


Figure 22: Observed and Chao1 alpha diversity measurements for all larval samples and their corresponding larval culture water samples (when collected). Mean alpha diversity values for the two sample types are shown in red. Each sample is coloured according to its collection date, with the first larval release being recorded on 03/08/2021.

A key aim of this chapter is to assess microbial differences in the microbiome of the larvae over time. NMDS analysis was therefore conducted, where data points (each representing one larval sample) were coloured according to their location of collection within the hatchery environment (tray or upwelling tank number); (Figure 23). Each data point was also assigned a shape, corresponding to their developmental larval stage at the time of collection. PERMANOVA statistical tests confirmed a significant difference between larvae of different larval stages, ($p = 0.02$, $r^2 = 0.245$), but not location ($p = 0.524$, $r^2 = 0.329$).

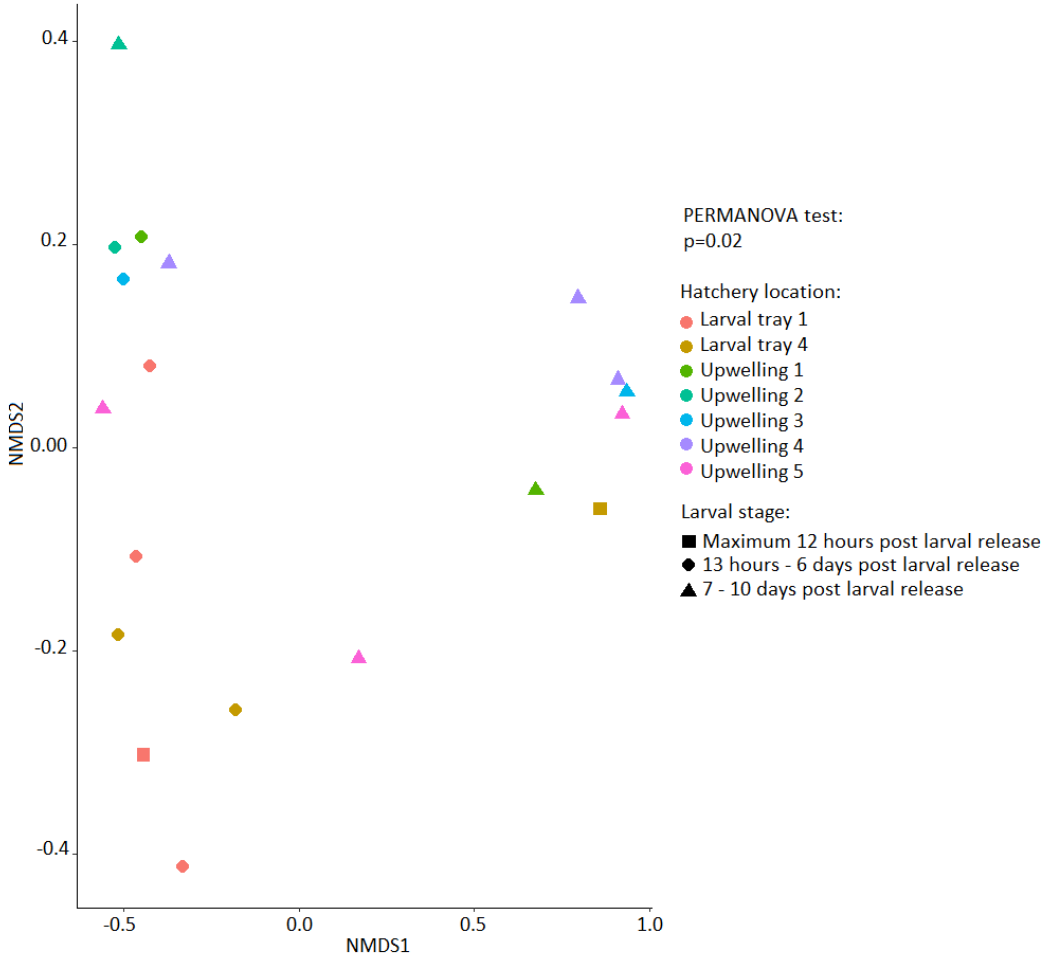


Figure 23: NMDS analysis of the 19 larval samples collected at Portsmouth. Data points are coloured according to their location of collection from the hatchery environment (larval collection or upwelling tank number) and assigned different shapes to denote their larval stage where (square = maximum 12 hours post larval release, circle = 13 hours – 6 days post larval release and square =7 – 10 days post larval release).

To further compare composition of microbial profiles of larvae at different developmental stages, alpha diversity measures were calculated in order to provide information on species richness between larvae at different developmental stages (see Figure 24). A wide variation was seen between alpha diversity measures of the two larval samples collected at a maximum of 12 hours post larval release, giving an average Observed and Chao1 values of 414.5 and 553.9. In larvae collected between 13 hours and 6 days post larval release, average Observed and Chao1 values then increased to 452.1 and 557.5. In larvae of the latter developmental stages (collected between 7-10 days post fertilisation), Observed and Chao1 alpha diversity values decreased to 334.3 and 439.4 in late veliger larvae (Figure 24). A Kruskal Wallis statistical test confirmed there to be no significant difference in alpha diversity measurements between larvae at these different developmental stages (Observed p = 0.7901; Chao1 p = 0.965).

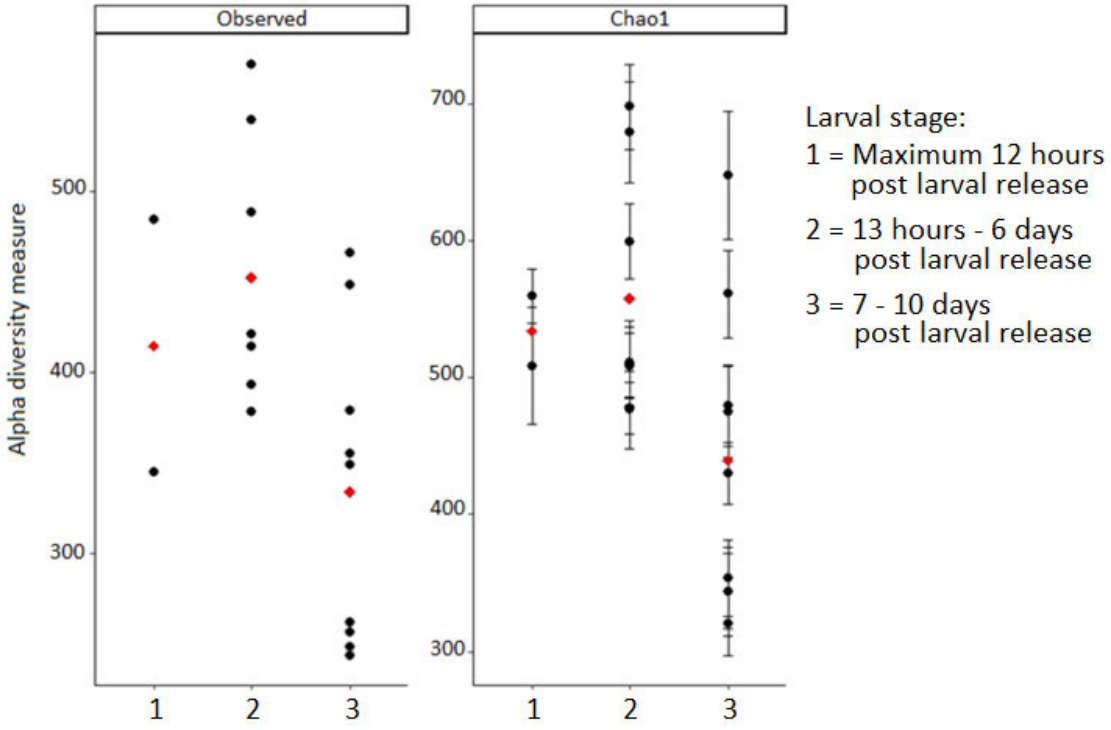


Figure 24: Observed and Chao1 alpha diversity indices for each larval sample. Larval samples are grouped according to their developmental stage (1 = maximum 12 hours post larval release, 2= 13 hours – 6 days post larval release and 3= 7 – 10 days post larval release).

Microbial profiles of larvae were compared to that of their immediate environment with alpha diversity measures calculated from samples of larval culture water (Figure 25). These are samples of water (containing no larvae) that the larvae shown in Figure 24 were reared in. Here, alpha average alpha diversity measures decreased over time as larvae progressed throughout the three developmental stages.

Average observed and Chao1 values for water surrounding larvae collected at a maximum of 12 hours post larval release were the highest at 608 and 791.1. These average values then decreased for culture water of larvae collected between 13 hours and 6 days post larval release with average observed and Chao1 values of 505.4 and 672.9. The lowest average alpha diversity measurements were seen within culture water of larvae collected between 7 and 10 days post larval release with Observed and Chao1 values of 427 and 561.3. A Kruskal Wallis statistical test confirmed there to be no significant difference in alpha diversity measurements between larval culture water samples from different larval developmental groups (Observed p = 0.7444; Chao1 p = 0.6585).

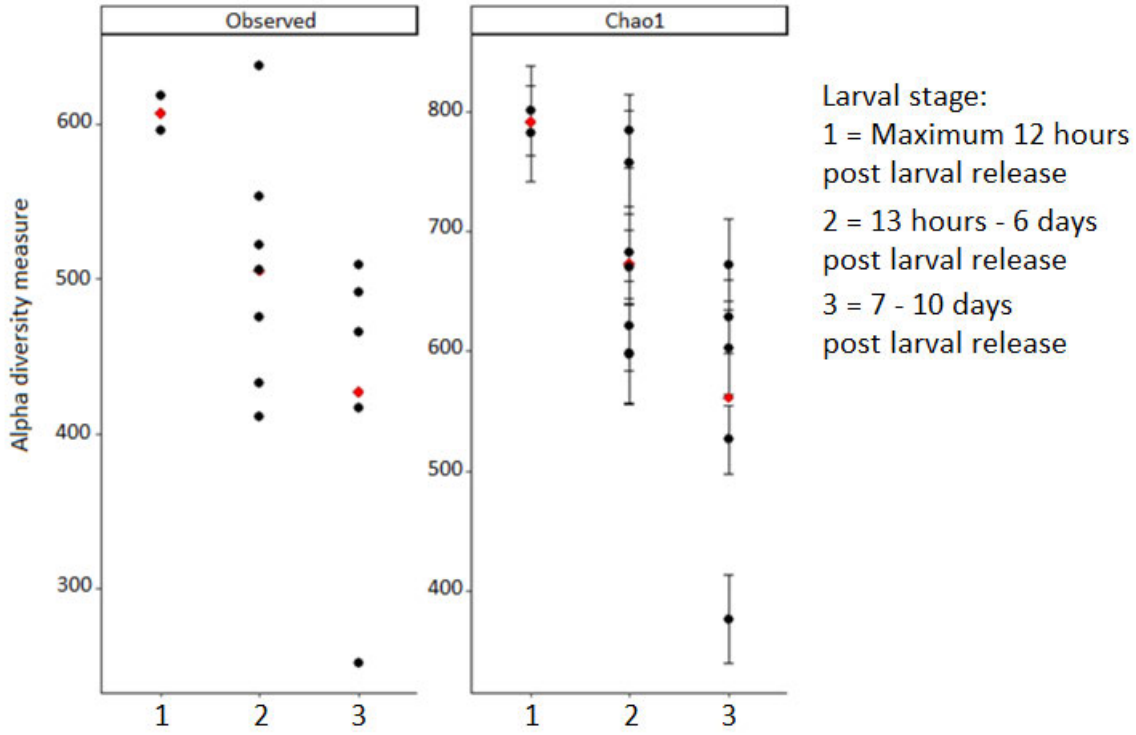


Figure 25: Observed and Chao1 alpha diversity measures for larval culture water samples (water containing no larvae). Water samples are grouped according to the developmental stage of their larvae (1 = maximum 12 hours post larval release, 2 = 13 hours – 6 days post larval release and 3 = 7 – 10 days post larval release).

Figures 26 and 27 below show the bacterial composition within each larval sample. A total of 23 phyla were identified, with Protobacteria being largely dominant in each larval sample, followed by Bacteroidota. Although not statistically significant, OTUs belonging to the Actinobacteriota phyla are also more relatively abundant in larvae sampled between 7 – 10 days post larval release (Figure 26).

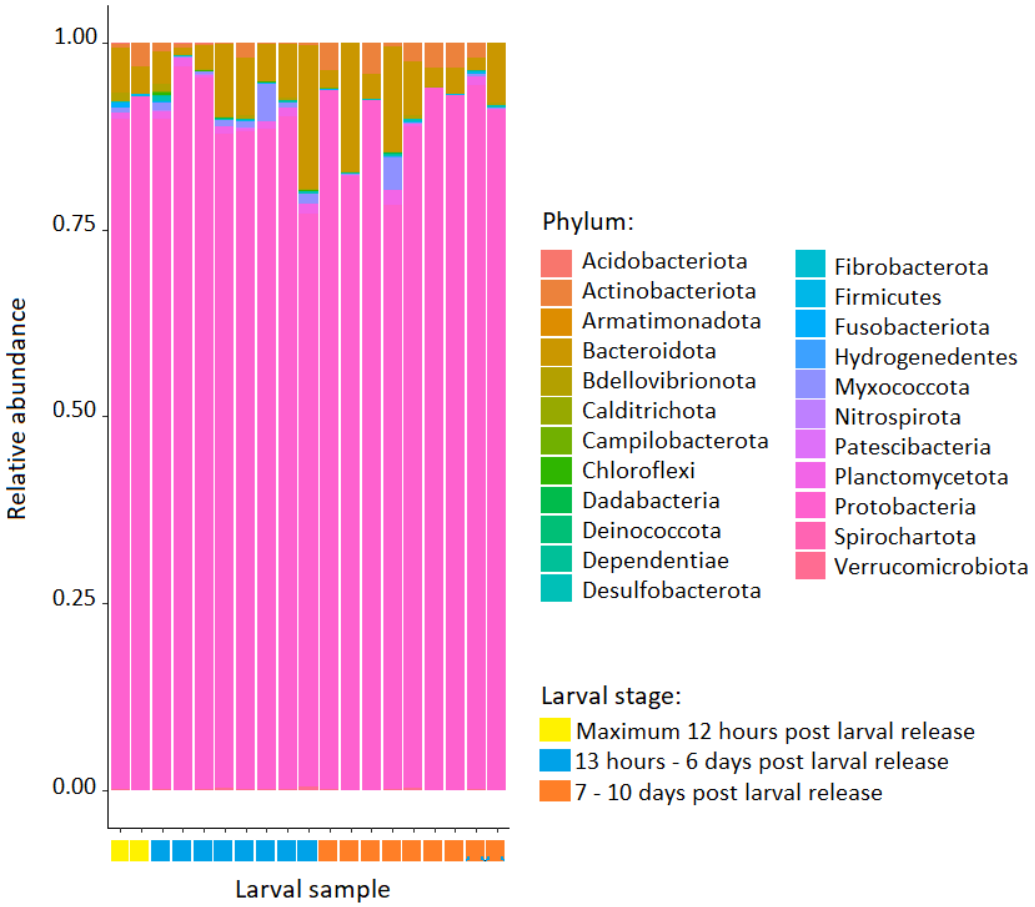


Figure 26: Bacterial composition of the 19 larval samples collected throughout from the Portsmouth hatchery. The relative abundance of 641 OTUs are shown according to their respective phyla. Annotations of yellow, blue and orange indicate the developmental stage of the larvae (maximum 12 hours post larval release, 13 hours – 6 days post larval release and 7 – 10 days post larval release.

The phyla shown in Figure 26 are represented in a heatmap form with Euclidean clustering being applied to both rows and columns (Figure 27). As previously seen, the two microbial samples collected at a maximum of 12 hours post larval release are clustered separately. between samples of trochophore larvae, with Acidobacteriota, Bdellovibrionota and Fusobacteriota being more dominant in one compared to the other. Larval samples collected between 13 hours and 6 days post larval release are generally clustered together and separately from those collected between 7 and 10 days post larval release, following the pooling of larvae. Larvae collected between 7 and 10 days post larval release had an over-represented relative abundances of Actinobacteriota, Firmicutes and Protobacteria in comparison to other larval stages (see Figure 27).

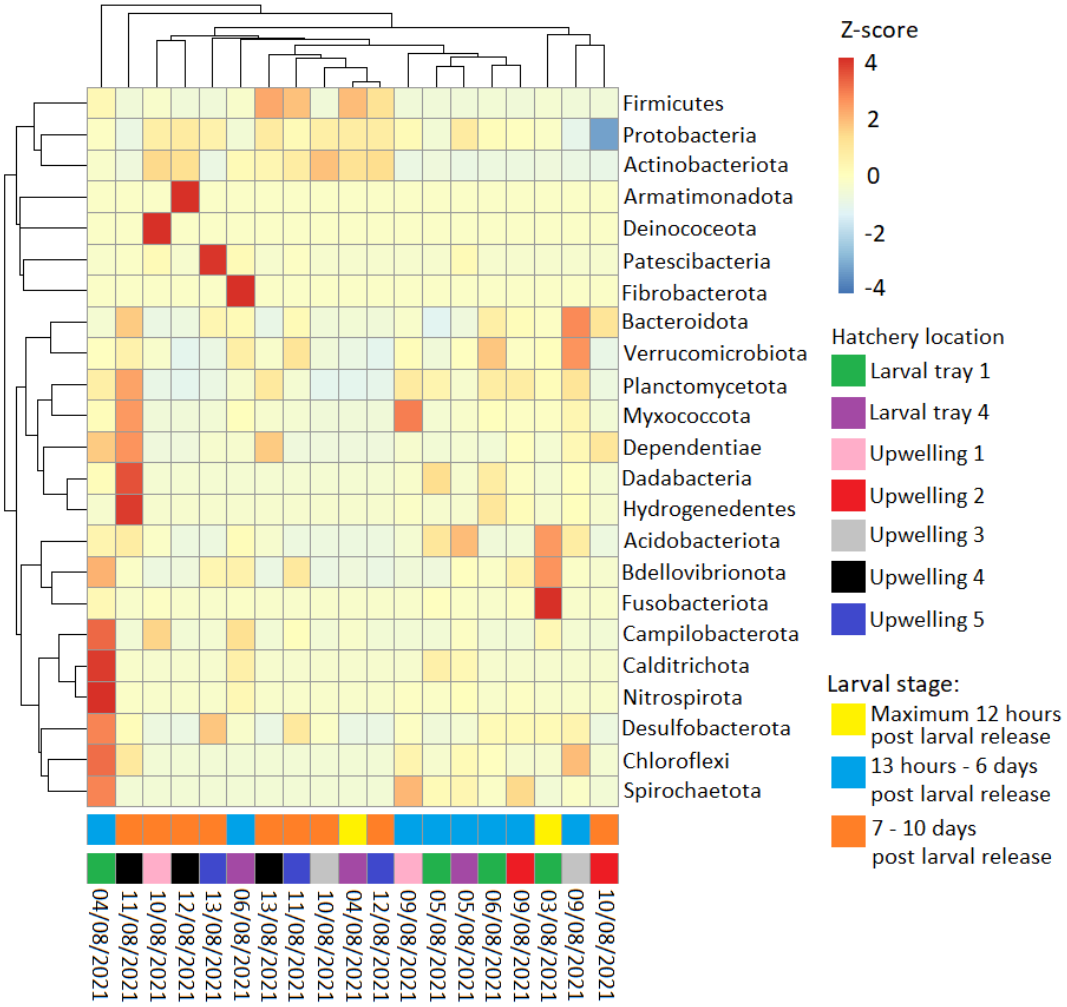


Figure 27: The relative abundance of the 23 bacterial phyla found throughout each larval sample. Relative abundance data was normalised to allow for expression in a Z-score format and Euclidean clustering applied to each row and column. Coloured annotations are used to denote the larval stage and collection point within the hatchery (tray or upwelling number) for each sample.

To identify taxa which statistically differed in their abundance throughout larval development, differential abundance analysis was conducted (Figure 28). Larvae collected between 13 hours and 6 days post larval release were treated as the baseline group and compared against those collected between 7 and 10 days post larval release. One OTU belonging to the *Limnohabitans* genus was significantly less abundant in the later developmental stage compared to the earlier. In contrast, nine OTUs were significantly more abundant in the later developmental stage compared to the earlier. These OTUs were either uncultured or unidentified at a genus level (NA), or belonged to *Synechocystis*, *Roseibacterium, Oceanicaulis*, *Dinoroseobacter* and *Arenicella*.

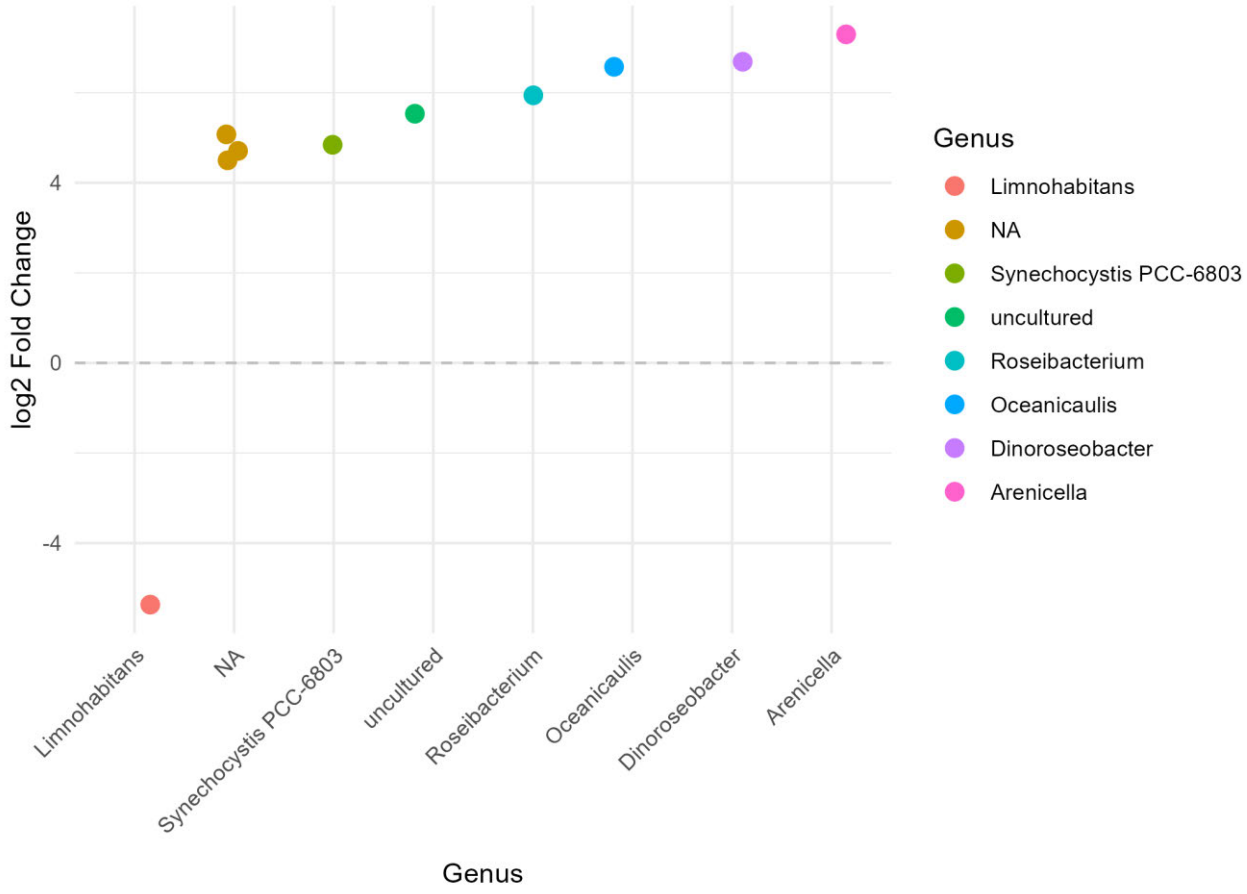


Figure 28: Differential abundance analysis of microbial taxa between larvae collected between 13 hours and 6 days post larval release, and 7 and 10 days post larval release. Each data point represents one OTU and is named according to its taxonomic classification at a genus level (where possible).

* * *

Aim 3: Investigate how hatchery processes specific to these locations including water filtration and algal cultivation methods influence microbial communities.

The bacterial composition of water samples collected from throughout the hatchery environment ('hatchery water') are shown in Figures 29 and 30. These are water samples collected throughout the hatchery process, including from the input source, through various filtration methods, and holding points within the hatchery. After filtering out taxa where the maximum proportion of reads per sample equalled less than 0.0001, a total of 777 OTUs were identified from hatchery water samples. The relative abundance of these 777 OTUs within each water sample are shown below, in terms of their respective phyla. In total, 29 different bacterial phyla were identified, with each sample being dominantly comprised of the Protobacteria phyla followed by Bacteroidota. Samples collected post filtration (header and broodstock tanks) have a lower relative abundance of OTUs belonging to the Nitrospinota, Dependentiae, Desulfobacterota, Elusimicrobiota, Verrucomicrobiota and Fibrobacterota phyla (Figure 29).

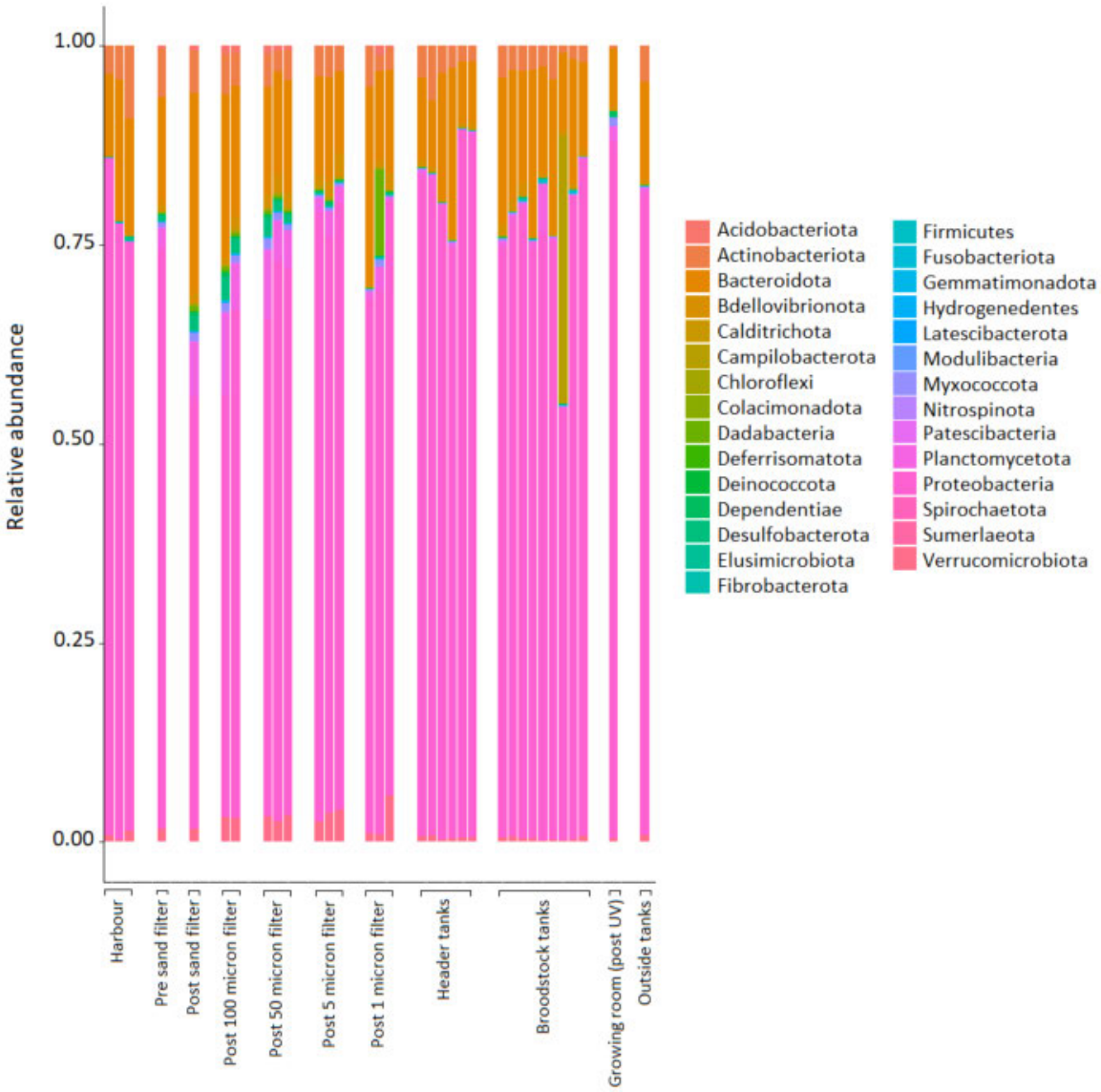


Figure 29: Relative abundances of the 23 different bacterial phyla found within water samples collected from throughout the Portsmouth hatchery environment. Water samples of the same type are shown in increasing date order.

The phyla shown in Figure 29 are represented in a heatmap format with Euclidean clustering (Figure 30). The abundance of Protobacteria shows little variation between samples. Samples collected from header and broodstock tanks (post filtration) show a lower relative abundance of the majority of phyla in comparison to samples collected prior to or throughout the filtration process. For example, water samples collected post 100 µm filter show a dominance of phyla such as Gemmatiminadota and Deferrisomatota which is not seen within water from header and broodstock tanks (Figure 30). The abundance of all phyla shown within water samples collected from the harbour appear with a Z- score value close to zero. It should be noted that since relative abundance values have been used, this should not be interpreted as water from the harbour containing a lower bacterial load overall.

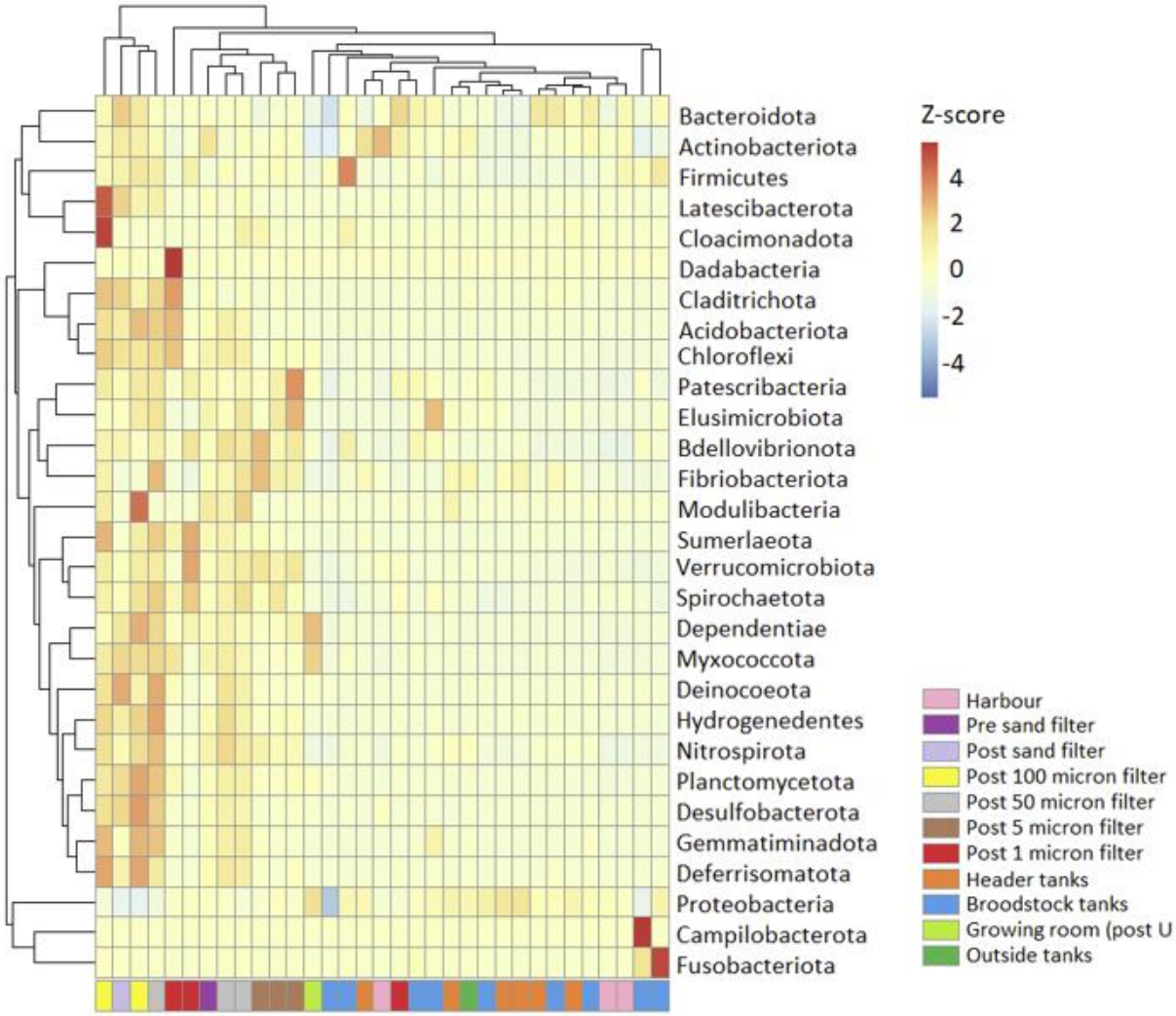


Figure 30: The relative abundance of the 29 bacterial phyla found throughout each hatchery water sample. Relative abundance data was normalised to allow for expression in a Z-score format and Euclidean clustering applied to each row and column. Coloured annotations indicate the hatchery location at from which the water sample was collected.

Differential abundance analysis was used to identify OTUs which statistically significantly differed in their abundance between pre and post water filtration. To do this, OTU abundances from samples collected pre sand filter from the harbour were used as a baseline and compared against those post filtration from either header tank one (Figure 31a) or header tank two (Figure 31b). The abundance of ten (Figure 31a) and eight (Figure 31b) OTUs were statistically lower in header tanks one and two retrospectively in comparison to the harbour.

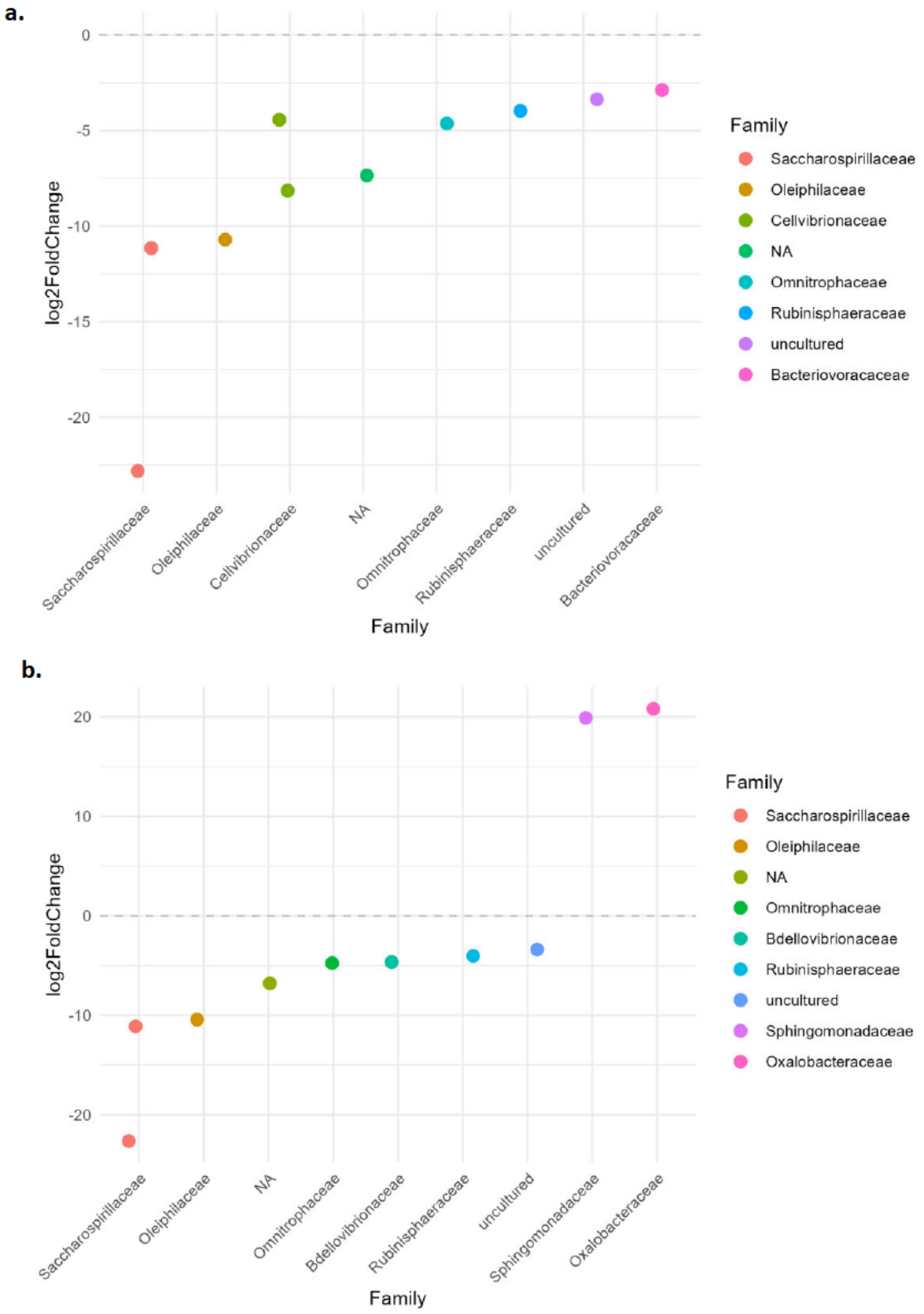


Figure 31: (a) Differential abundance analysis of microbial taxa between water samples collected from the harbour (pre filtration) and header tank one (post filtration). **(b)** Differential abundance analysis of microbial taxa between water samples collected from the harbour (pre filtration) and header tank two (post filtration). Each data point represents one OTU and is named according to its taxonomic classification at a family level (where possible). OTUs which were not identified at a family level were assigned the label 'NA'.

The bacterial compositions of algal and neutralised sea water samples collected are also shown below (Figures 32 and 33). Nine different phyla were identified within these samples. The Protobacteria phylum was the most dominant in all four samples, followed by the Actinobacteriota phylum. Although not statistically significant, algal samples contained a higher proportion of the Firmicutes phylum compared to neutralised sea water, whereas Verrucomicrobiota were only present in neutralised sea water (see Figure 32).

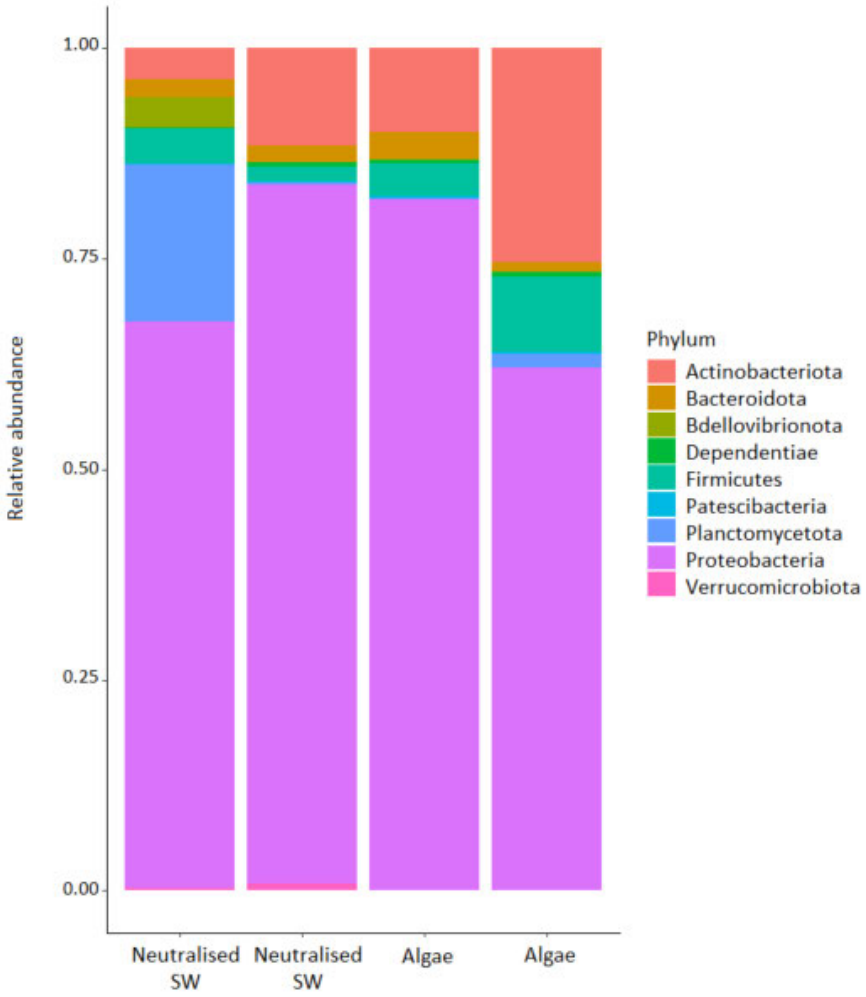


Figure 32: The bacterial composition of algae and neutralised sea water samples. The neutralisation of sea water was carried out as a stage of algal production. Relative abundance data for the nine bacterial phyla detected with each sample is show.

A heatmap showing the relative abundance of the nine bacterial phyla found across neutralised seawater and algal samples is shown below (Figure 33). The relative abundance of these bacterial phyla are more consistent in algal samples compared to Neutralised seawater, although Firmicutes and Patescibacteria are dominant in one algal sample.

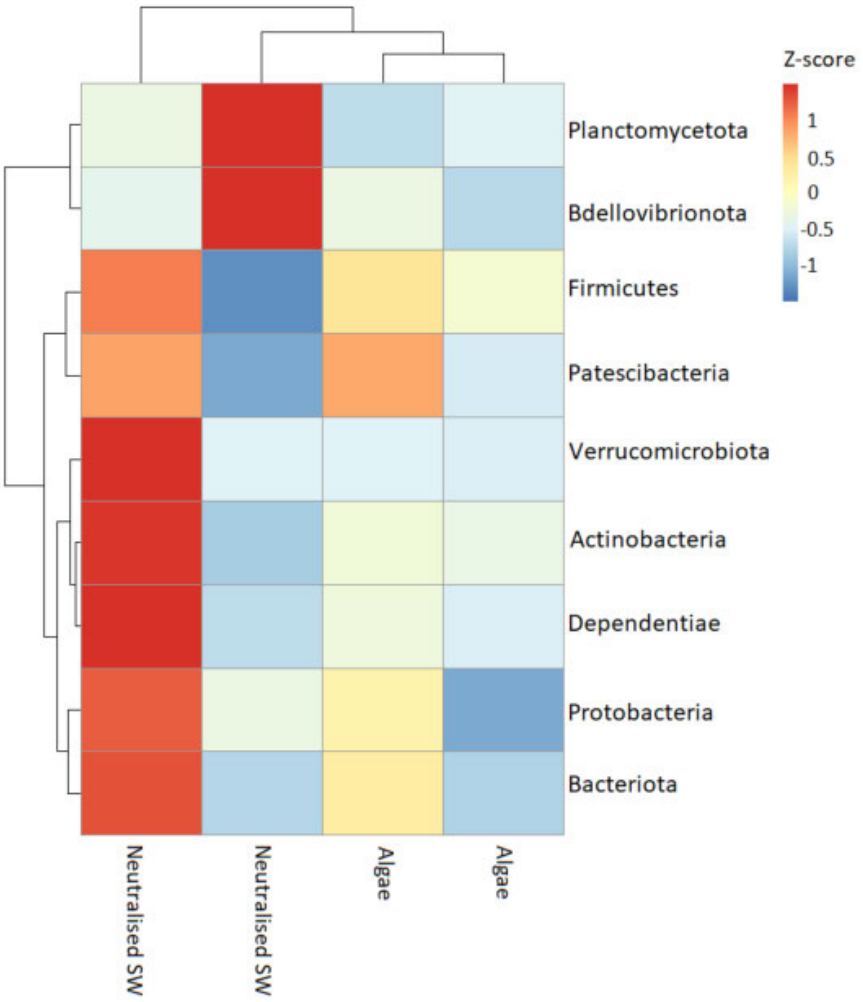
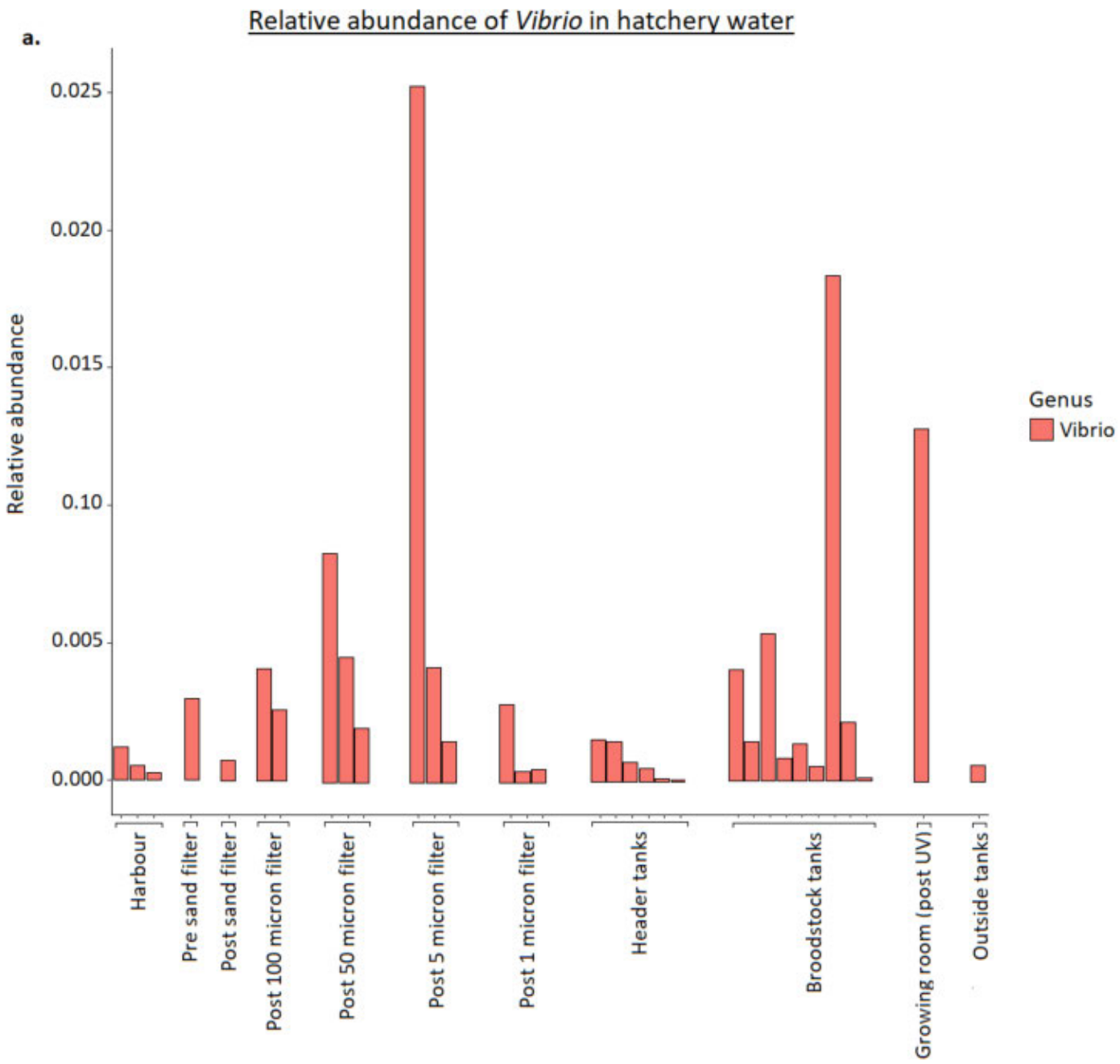
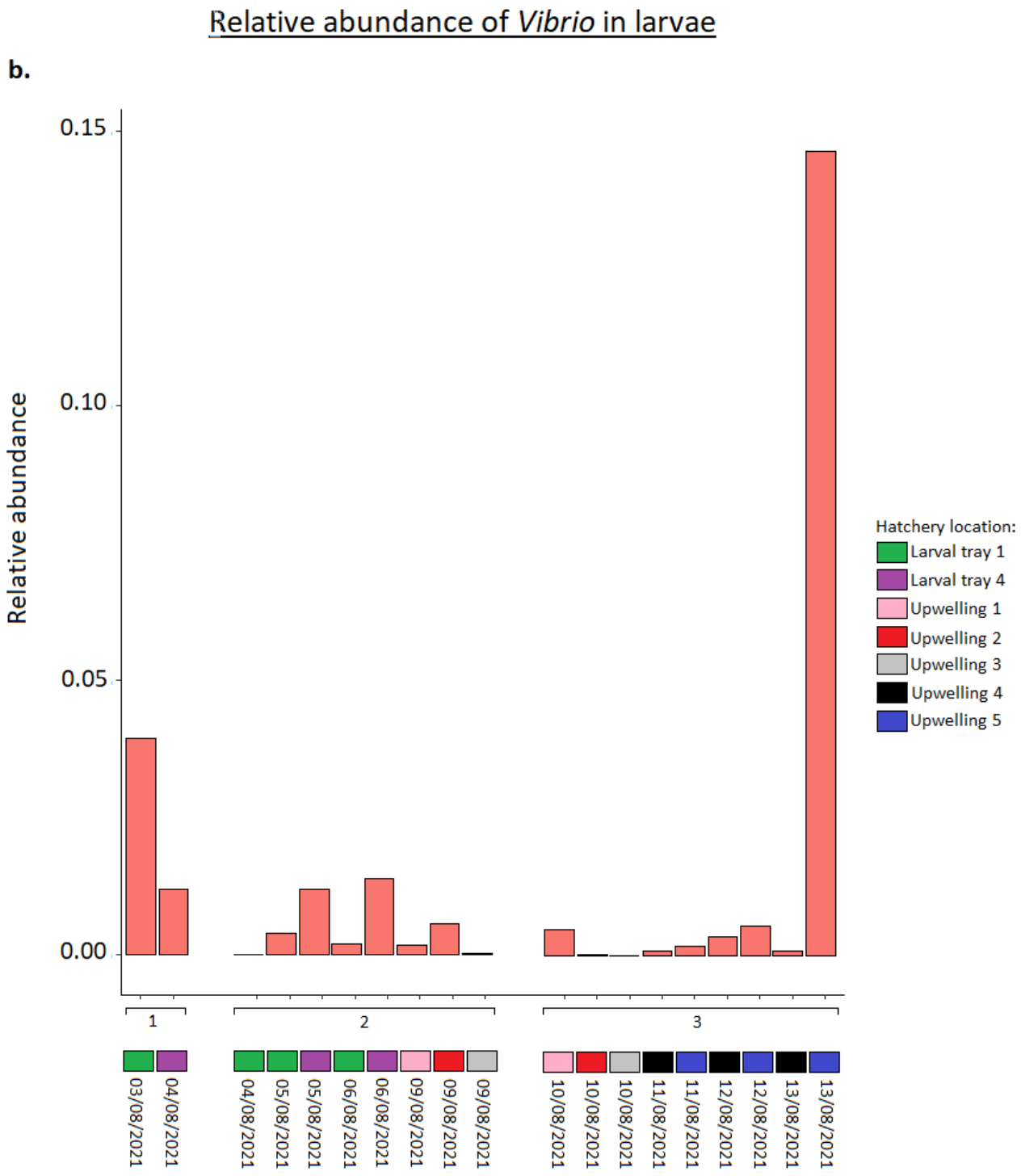


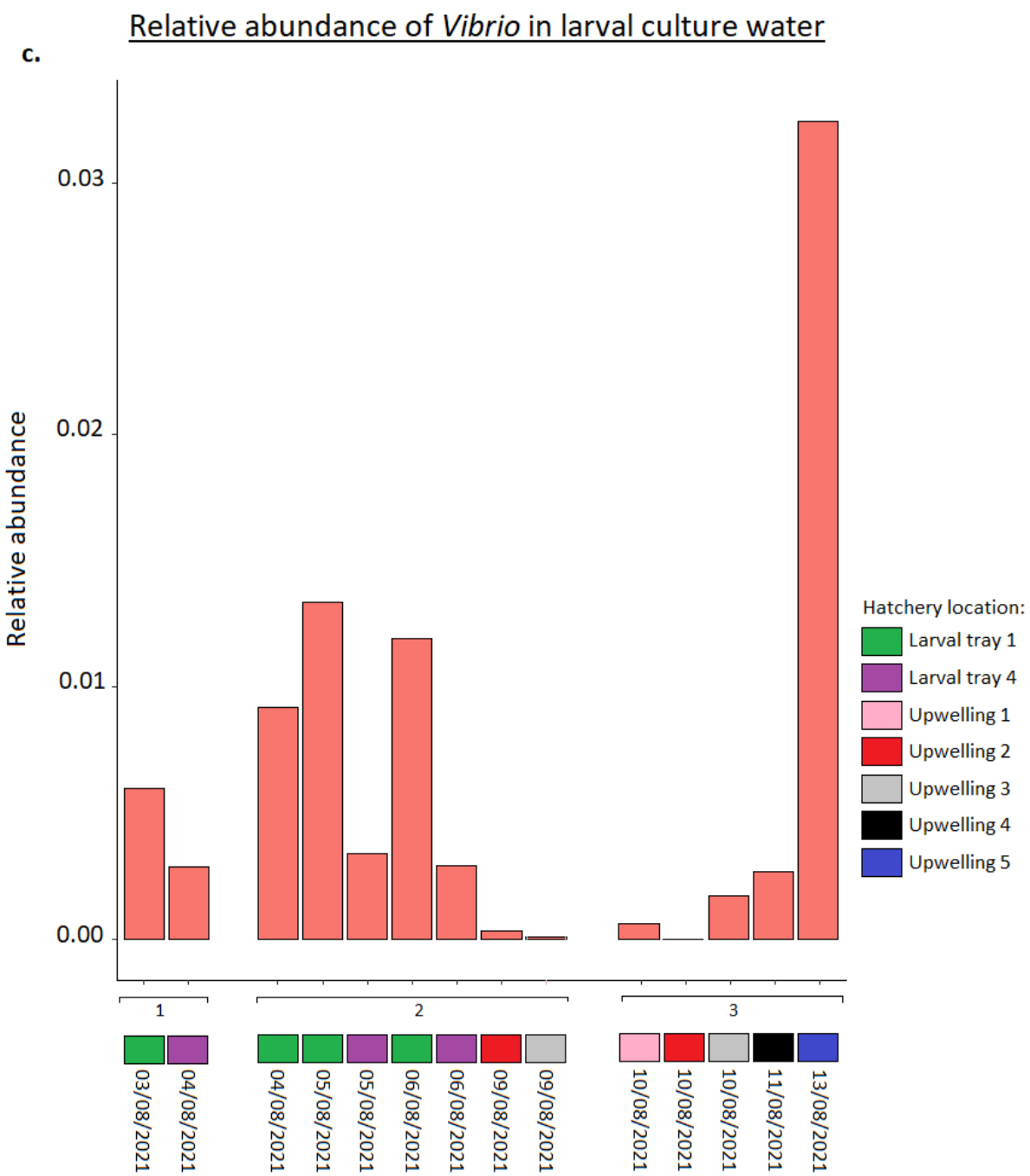
Figure 33: Relative abundance of the nine bacterial phyla found in algal and neutralised sea water samples. Relative abundance data was normalised to allow for expression in a Z-score format and Euclidean clustering applied to each row and column.

A single OTU was identified as belonging to the *Vibrio* genus. The relative abundance levels of this OTU are shown across sample types of hatchery water (Figure 34a), larvae (Figure 34b), larval culture water (Figure 34c), algae (Figure 34d) and pallial cavity water (Figure 34e). The relative abundance of *Vibrio* is generally highest in larval samples, followed by samples of larval culture water.

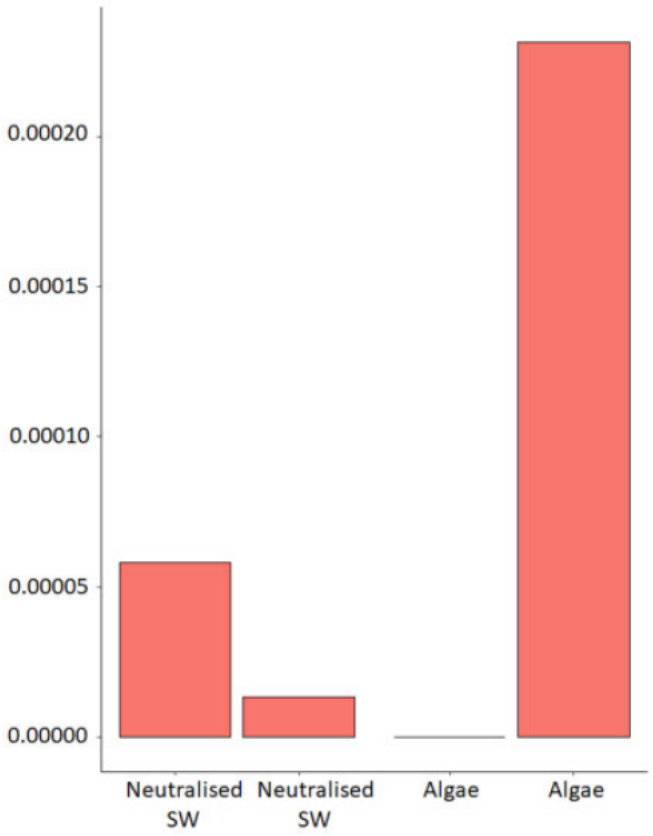
The relative abundance of *Vibrio* increases throughout the different water filtration processes, before becoming more consistent in water samples from header and broodstock tanks (Figure 33a). The highest relative abundance of *Vibrio* amongst larval samples was collected from upwelling tank 5 on 13/08/2021 (Figure 34b). Similarly, the highest relative abundance of *Vibrio* amongst larval culture water samples corresponded to this, also being collected from upwelling tank 5 on 13/08/2021 (Figure 34c). In comparison, samples of algae, neutralised seawater and pallial cavity water show the lower relative abundance levels of *Vibrio* (Figures 34d and 34e).


a.
Relative abundance of *Vibrio* in hatchery water
Relative abundance
0.025
0.020
0.015
0.10
0.005
0.000
Harbour
Pre sand filter
Post sand filter
Post 100 micron filter
Post 50 micron filter
Post 5 micron filter
Post 1 micron filter
Header tanks
Broodstock tanks
Growing room (post UV)
Outside tanks
Genus
Vibrio


Relative abundance of *Vibrio* in larvae
b.
Relative abundance
0.15
0.10
0.05
0.00
1
2
3
03/08/2021
04/08/2021
04/08/2021
05/08/2021
05/08/2021
06/08/2021
06/08/2021
09/08/2021
09/08/2021
09/08/2021
10/08/2021
10/08/2021
10/08/2021
11/08/2021
11/08/2021
12/08/2021
12/08/2021
13/08/2021
13/08/2021
Hatchery location:
Larval tray 1
Larval tray 4
Upwelling 1
Upwelling 2
Upwelling 3
Upwelling 4
Upwelling 5


c.
Relative abundance of *Vibrio* in larval culture water
Relative abundance
0.03
0.02
0.01
0.00
1
2
3
03/08/2021
04/08/2021
04/08/2021
05/08/2021
05/08/2021
06/08/2021
06/08/2021
09/08/2021
09/08/2021
10/08/2021
10/08/2021
10/08/2021
11/08/2021
13/08/2021
Hatchery location:
Larval tray 1
Larval tray 4
Upwelling 1
Upwelling 2
Upwelling 3
Upwelling 4
Upwelling 5

d. Relative abundance of *Vibrio* in algae



e. Relative abundance of *Vibrio* in pallial cavity water

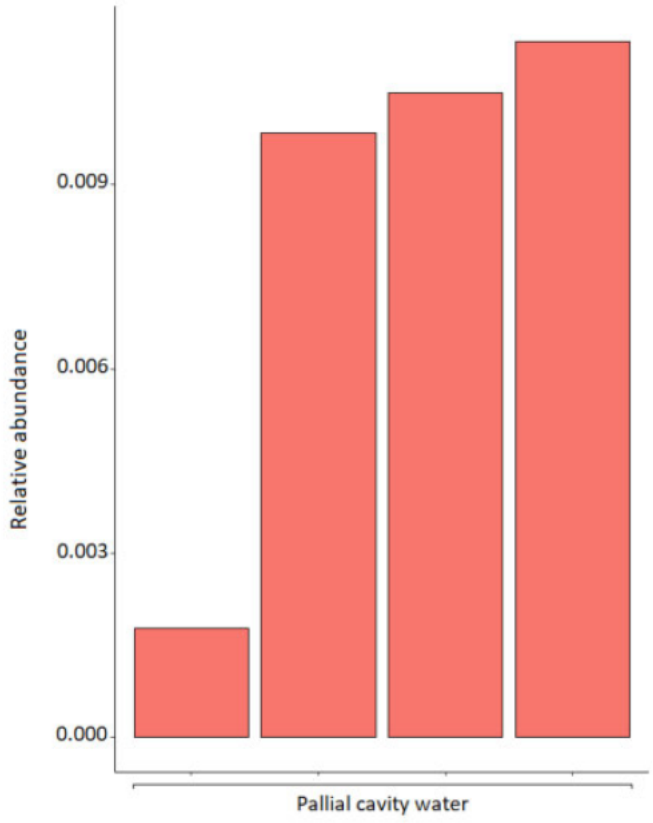


Figure 34: Relative abundance profile of the *Vibrio* genus throughout different sample types including **(a)** hatchery water **(b)** larvae **(c)** larval culture water **(d)** algae **(e)** and pallial cavity water. Samples of larvae and larval culture water are annotated according to their time of collection in days post larval release where 1 = maximum of 12 hours post larval release, 2= 13 hours – 6 days post larval release and 3 = 7 – 10 days post larval release. Each sample is also annotated with its location of collection within the hatchery (larval tray or upwelling tank number) and date of collection.

* * *

Results (Morecambe Bay):

Aim 1: To identify core larval taxa and how these change throughout the rearing process.

A total of 883 OTUs were identified within the 24 larval samples collected at Morecambe Bay. A with the Portsmouth University hatchery, OTUs which were present in all larval samples from Morecambe Bay were selected to identify core bacterial taxa of these larvae. From the original 883 OTUs identified, 41 were present in each of the larval samples and have therefore been used to describe the core larval microbiome. The relative abundance of these OTUs across each larval sample is shown below, with taxonomic identifications given at a genus level (Figure 35). In instances where OTUs which could not be identified to a genus level, identification is given at a family (*) or order level (**).

Using these core OTUs, larval samples appear in two main clusters, which share a mix of early and late veliger samples. D-shaped larvae (blue), cluster together and show a higher relative abundance of the majority of core taxa (highlighted via the red box). This clustering pattern is also shared by five out of the seven early veliger samples collected between three and seven days post fertilisation. By comparison, pediveliger larvae show a lower relative abundance of these core taxa (highlighted via the red box), and a higher relative abundance of those highlighted via the green box - belonging to genera such as *Erythrobacter*, *Paracoccus*, *Silicimonas*, *Tateyamaria* and *Rhodobacteraceae* (in comparison to their overall mean). This clustering pattern is shared by four out of the 12 late veliger samples which were collected between 21 and 24 days post fertilisation.

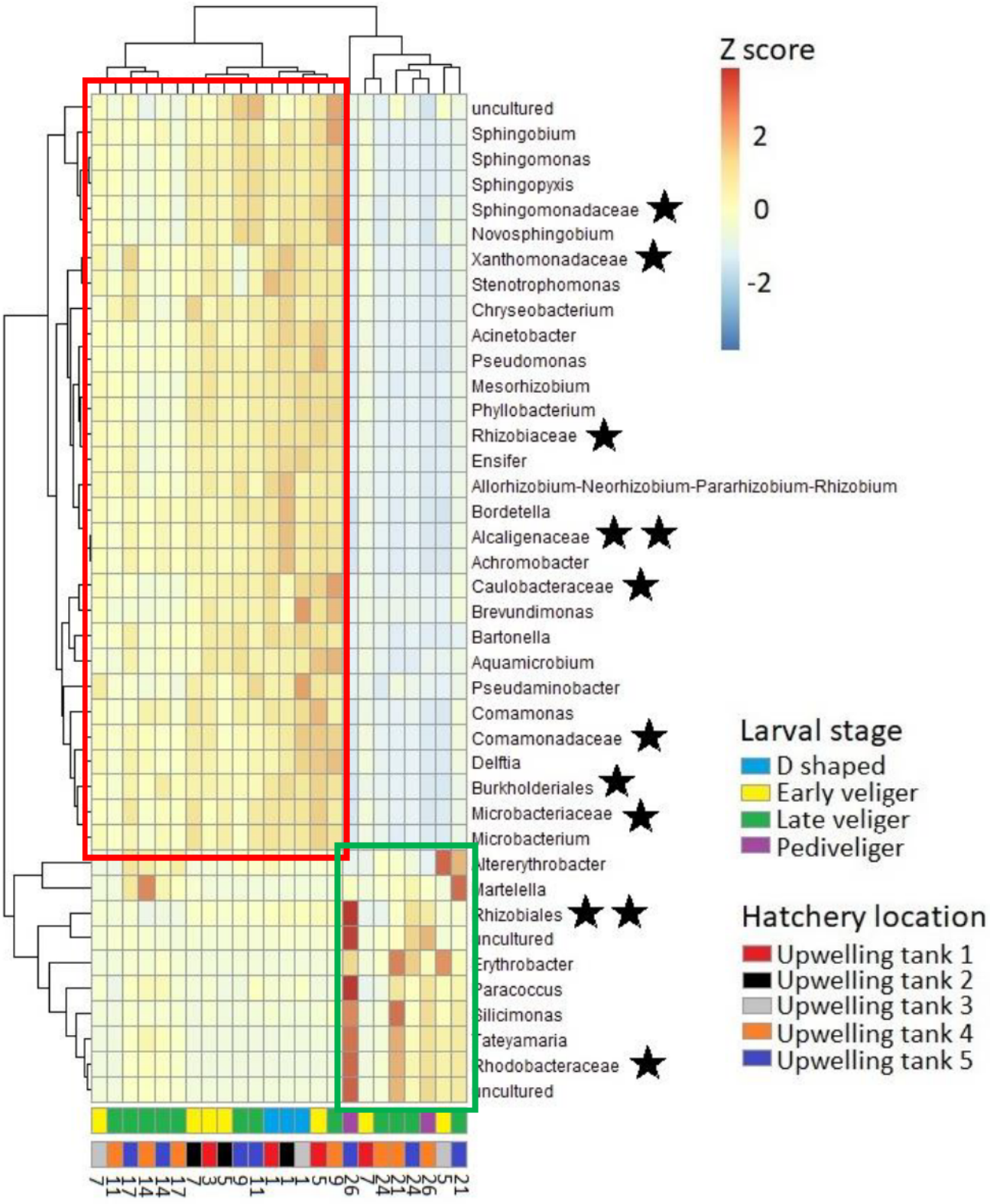

Z score
2
0
-2
uncultured
Sphingobium
Sphingomonas
Sphingopyxis
Sphingomonadaceae
Novosphingobium
Xanthomonadaceae
Stenotrophomonas
Chryseobacterium
Acinetobacter
Pseudomonas
Mesorhizobium
Phyllobacterium
Rhizobiaceae
Ensifer
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Bordetella
Alcaligenaceae
Achromobacter
Caulobacteraceae
Brevundimonas
Bartonella
Aquamicrobium
Pseudaminobacter
Comamonas
Comamonadaceae
Delftia
Burkholderiales
Microbacteriaceae
Microbacterium
Rhizobiales
Rhodobacteraceae
Larval stage
D shaped
Early veliger
Late veliger
Pediveliger
Hatchery location
Upwelling tank 1
Upwelling tank 2
Upwelling tank 3
Upwelling tank 4
Upwelling tank 5

Figure 35: A heatmap of the 41 OTUs present in all 24 larval samples collected throughout the Morecambe Bay strip spawning event. Each sample is annotated with its corresponding larval stage, hatchery location and collection date in days post spawning. D-shaped larvae were collected between 12 and 24 hours post fertilisation. Early, late and pediveliger larvae were collected between 3-7, 9-24 and 26-days post fertilisation. Upwelling tanks 1, 2 and 3 were pooled at 9 days and redistributed into tanks 4 and 5. Z-score normalisation was performed on relative abundance data and Euclidean clustering applied to rows and columns. Each OTU is identified down to a genus level unless annotated with '*' or '**' symbols, indicating identification at a family or order level. Red and green boxes are used to annotate OTUs groupings of particular interest.

* * *

Aim 2: Compare bacterial profiles of larvae to those of their hatchery environment.

In order to visulalise microbial similarities between the different sample types collected throughout this spawning event, NMDS analysis was conducted on all OTUs from the complete sample set (Figure 36). Each data point is representitive of one sample, with colour denoting the sample type and shape denoting the larval stage. Larval samples (dark blue) appear in two clear groupings, closest to either biofilm or hatchery water samples. PERMANOVA statistical tests reported a significant difference between different sample types ($p = 0.001$, $r^2 = 0.323$) and variying larval stages ($p = 0.009$, $r^2 = 0.080$). Microbial differences between samples are therefore driven by both sample type and stage of larval development (Figure 36). With consideration of the r^2 values reported above, variation in sample type was responsible for a greater proportion of microbial variability between samples than larval stage.

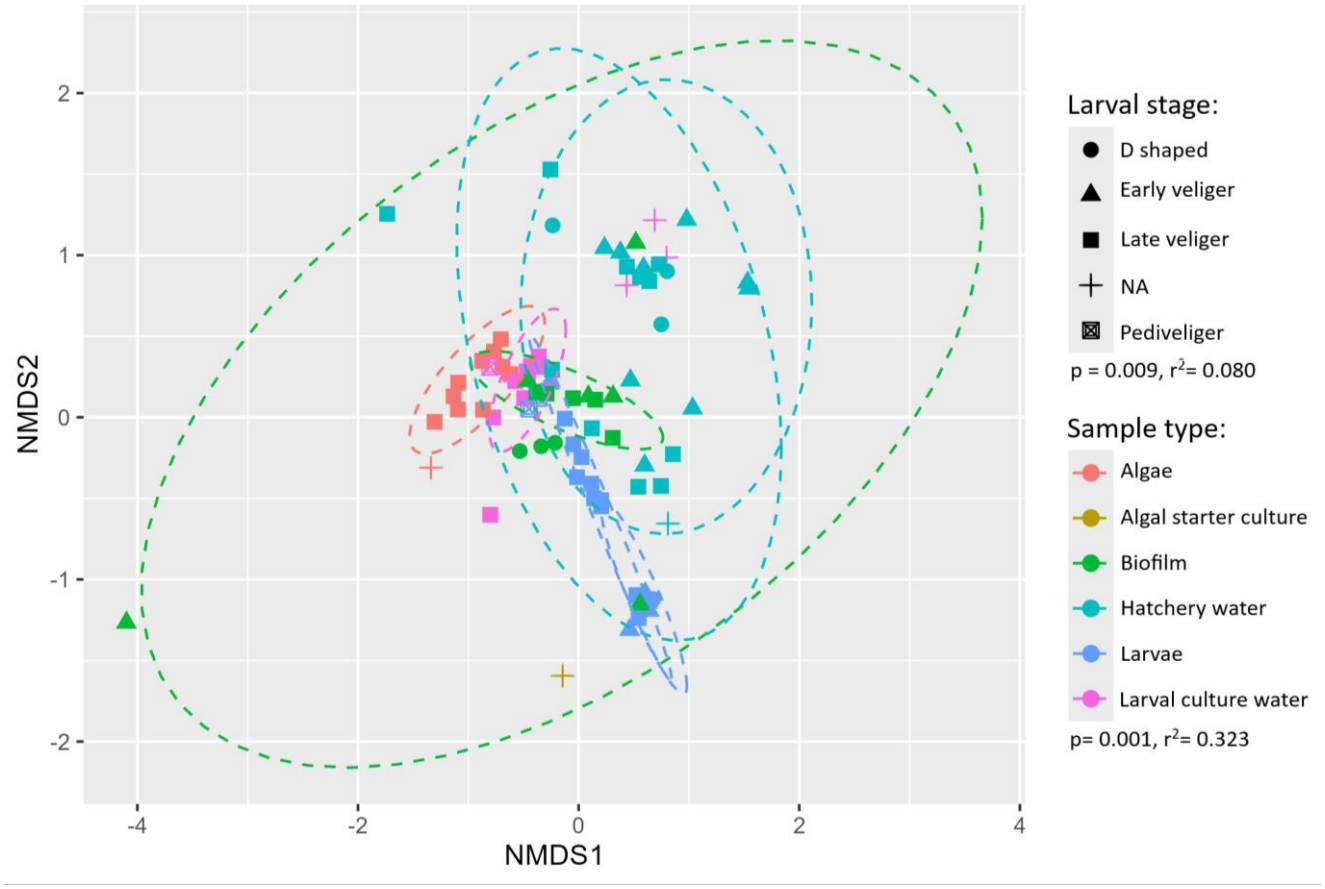


Figure 36: NMDS analysis conducted on all of the 88 samples collected from Morecambe Bay, where each datapoint represents one sample. Data points are coloured according to sample type covering algae, algal starter cultures, hatchery water, biofilm, larvae and larval culture water. The shape of each datapoint indicates the life stage of larvae at the time of sample collection. D-shaped larvae are defined as larvae collected between 12-24 hours post fertilisation. Early and late veliger larvae were collected between 3-7 days and 9-24 days post fertilisation respectively. Pediveliger larvae were collected at 26 days post fertilisation and samples given an 'NA' larval stage category were collected prior to the addition of larvae to upwelling tanks. Ellipses indicate a 95% confidence limit around each sample type.

Possible reasonings for the two clear groupings of larval samples seen in Figure 34 were explored further through alpha diversity analysis. Levels of species richness within larval microbiomes are presented below with samples categorised into their developmental stage (Figure 37). Average species richness levels were higher in larvae of the later developmental stages (late veliger and pediveliger larvae). Species richness levels between developmental stages were however not statistically significant with a Kruskal-Wallis test reporting Observed and Chao1 p values of 0.6085 and 0.7793.

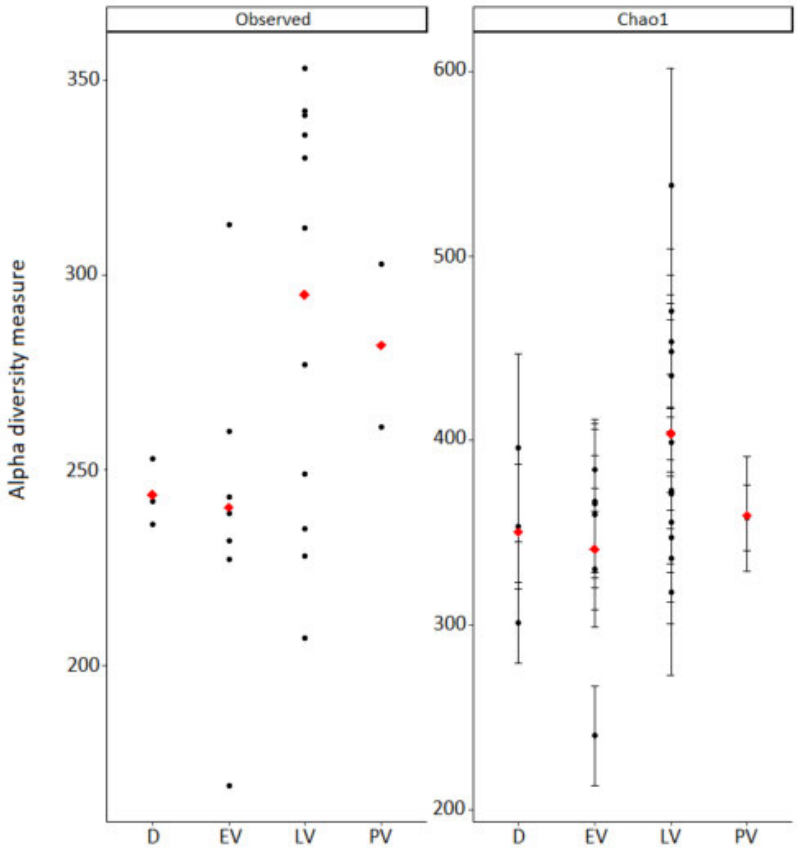


Figure 37: Observed and Chao1 alpha diversity measures of larval samples collected throughout the Morecambe Bay strip spawning event. D-shaped larvae are defined as larvae collected between 12-24 hours post fertilisation. Early and late veliger larvae were collected between 3-7 days and 9-24 days post fertilisation respectively. Pediveliger larvae were collected at 26 days post fertilisation. Mean alpha diversity values for each group are shown in red.

Alpha diversity analysis was also conducted on samples of larval culture water - again separated into the developmental stage of larval cultures from which they were collected (Figure 38). Average species richness levels were highest in larval culture water which was collected prior to the adding of any larvae (annotated as 'NA'). Average species richness was also higher in late veliger larvae compared to pediveligers (although only one pediveliger sample was collected). A Kruskal Wallis test reported there to be no statistical difference between species richness between these different groupings of larval culture water (Observed p = 0.4039, Chao1 p = 0.4989).

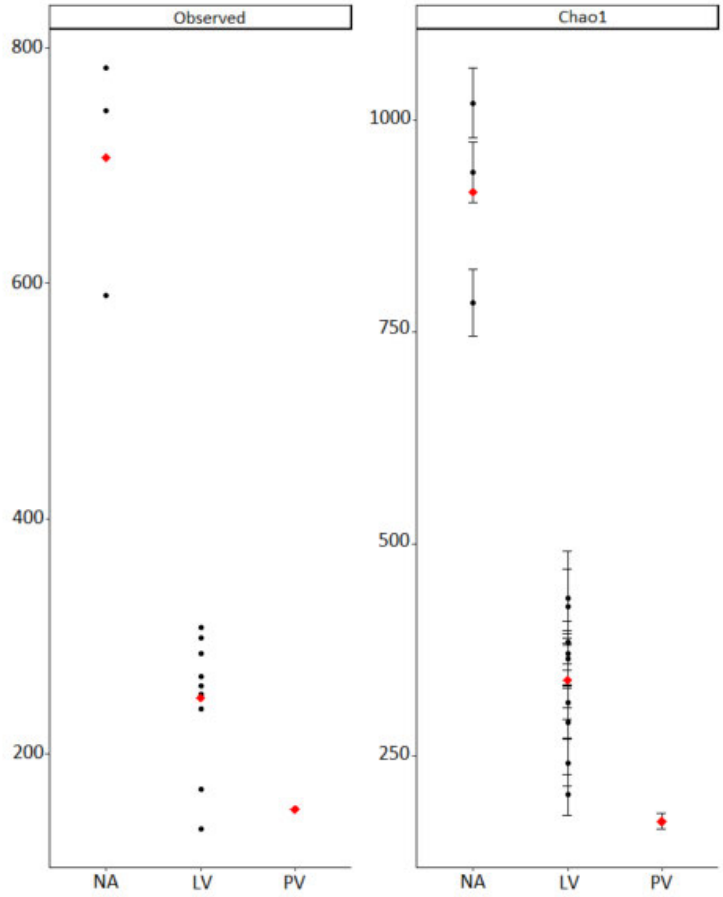


Figure 38: Observed and Chao1 alpha diversity measures of larval culture water samples collected throughout the Morecambe Bay strip spawning event. Water samples are shown grouped according to the developmental stage of larvae from which they were taken. Samples grouped as 'NA' represent water from upwelling tanks taken prior to the addition of larvae. Late veliger and pediveliger larvae were collected 9 – 24 days and 26 days post fertilisation. Mean alpha diversity values for each group are shown in red.

Initially, 833 OTUs were detected across all larval samples. These OTUs were filtered so that taxa where the maximum proportion of reads per sample equalled less than 0.0001 were removed, leaving a total of 336 OTUs. These 336 OTUs were assigned to 13 different bacterial phyla, the proportions of which across each larval sample are shown below (Figure 39). The majority of each larval sample is composed of the Protobacteria phyla, followed by Actinobacteriota and Bacteroidota. The relative abundance of Bacteroidota and Myxococcota increases with time within the late veliger larval stages, whereas the relative abundance of Actinobacteriota decreases. The relative abundance of *Bacteroidota* and Myxococcota decrease further still within pediveliger larvae, with Actinobacteriota becoming completely absent (Figure 38). This observations are a descriptive summary, and it should be noted that the statistical significance of variations in these taxa between larval samples is not reported upon.

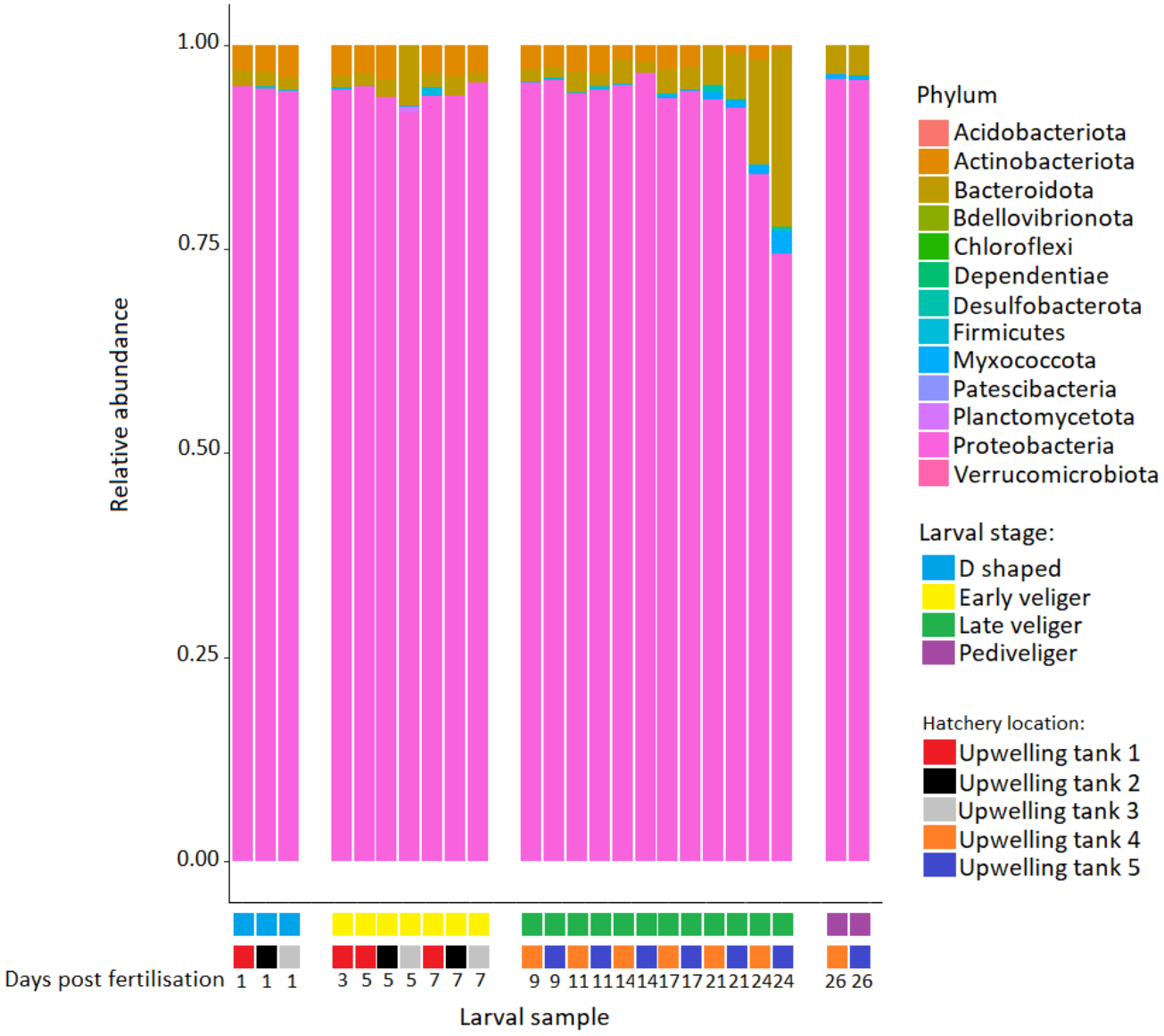


Figure 39: Bacterial composition of the 24 larval samples collected from Morecambe Bay hatchery. The relative abundance of 336 OTUs are shown according to their respective phyla. Annotations of yellow, blue and orange and indicate the developmental stage of the larvae (D-shaped larvae, early veliger, late veliger and pediveliger). D-shaped larvae are defined as larvae collected between 12-24 hours post fertilisation. Early and late veliger larvae were collected between 3-7 days and 9-24 days post fertilisation respectively. Pediveliger larvae were collected at 26 days post fertilisation. Further annotations show the hatchery location and days post fertilisation at which the larval sample was collected.

This bacterial composition within larval samples was explored further through representation in a heatmap with relative abundance values expressed in a Z-score and Euclidean clustering applied to rows and columns (see Figure 40). Whilst the profiles of all these core phyla are similar, with no clear clustering of samples there are some small differences. Out of the seven early veliger samples of larvae, four are grouped together, with relative abundances of either Planctomycetota, Acidobacteriota or Dependentiae being higher in these samples in comparison to their overall mean. Older larvae belonging to the late veliger group clustered on the right-hand side have a higher relative abundance of Patescibacteria, Bacteroidota, Myxococcota, Bdellovibrionota and Desulfobacterota in comparison to the overall mean values for these phyla (Figure 40). Again, it should be noted that the statistical significance of these observations is not reported upon.

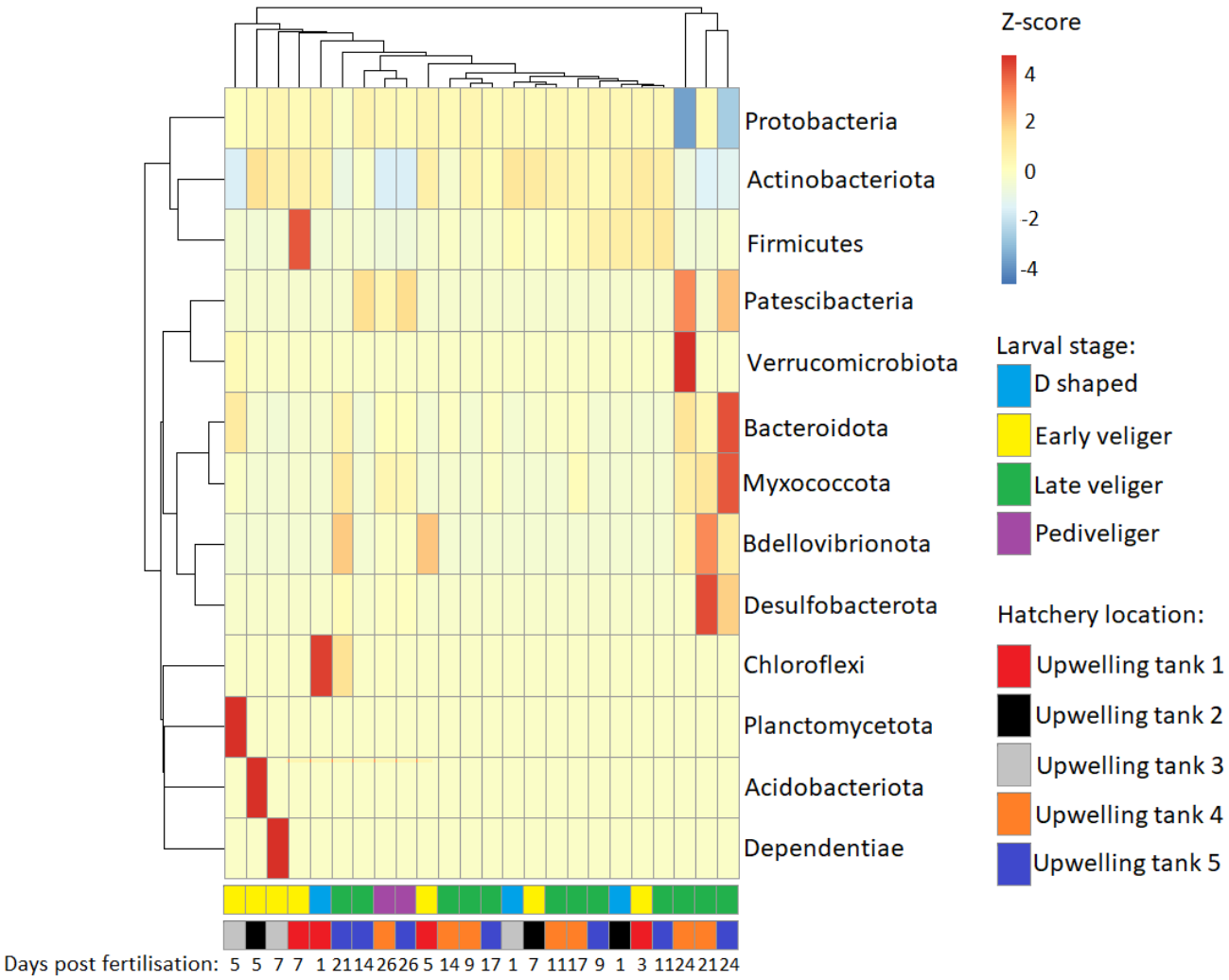


Figure 40: Heatmap showing relative abundance data of bacterial phyla present within all larval samples collected throughout the strip spawning event at Morecambe Bay. Abundance values have been normalised for expression as Z score values and Euclidean clustering applied to each row and column. Column annotations indicate the developmental stage at which the larval sample was collected (yellow = D-shaped, blue = early veliger, orange = late veliger and green = pediveliger). D-shaped larvae are defined as larvae collected between 12-24 hours post fertilisation. Early and late veliger larvae were collected between 3-7 days and 9-24 days post fertilisation respectively. Pediveliger larvae were collected at 26 days post fertilisation. Further annotations show the hatchery location and days post fertilisation at which the larval sample was collected.

* * *

Aim 3: Investigate how hatchery processes specific to these locations including water filtration and algal cultivation methods influence microbial communities.

A total of 28 different bacterial phyla were found within hatchery water samples (see Figure 41). The majority of each sample is primarily comprised of bacteria belonging to the Proteobacteria phylum, followed by Bacteroidota and Actinobacteriota. Samples collected within the hatchery building, but before UV treatment have a greater relative abundance of bacteria belonging to Depedentiae, Desulfobacterota, Fusobacteriota and Nitrospinota phyla. Figure 41 shows this level of diversity to be reduced in UV treated samples (from broodstock and larval header tanks).

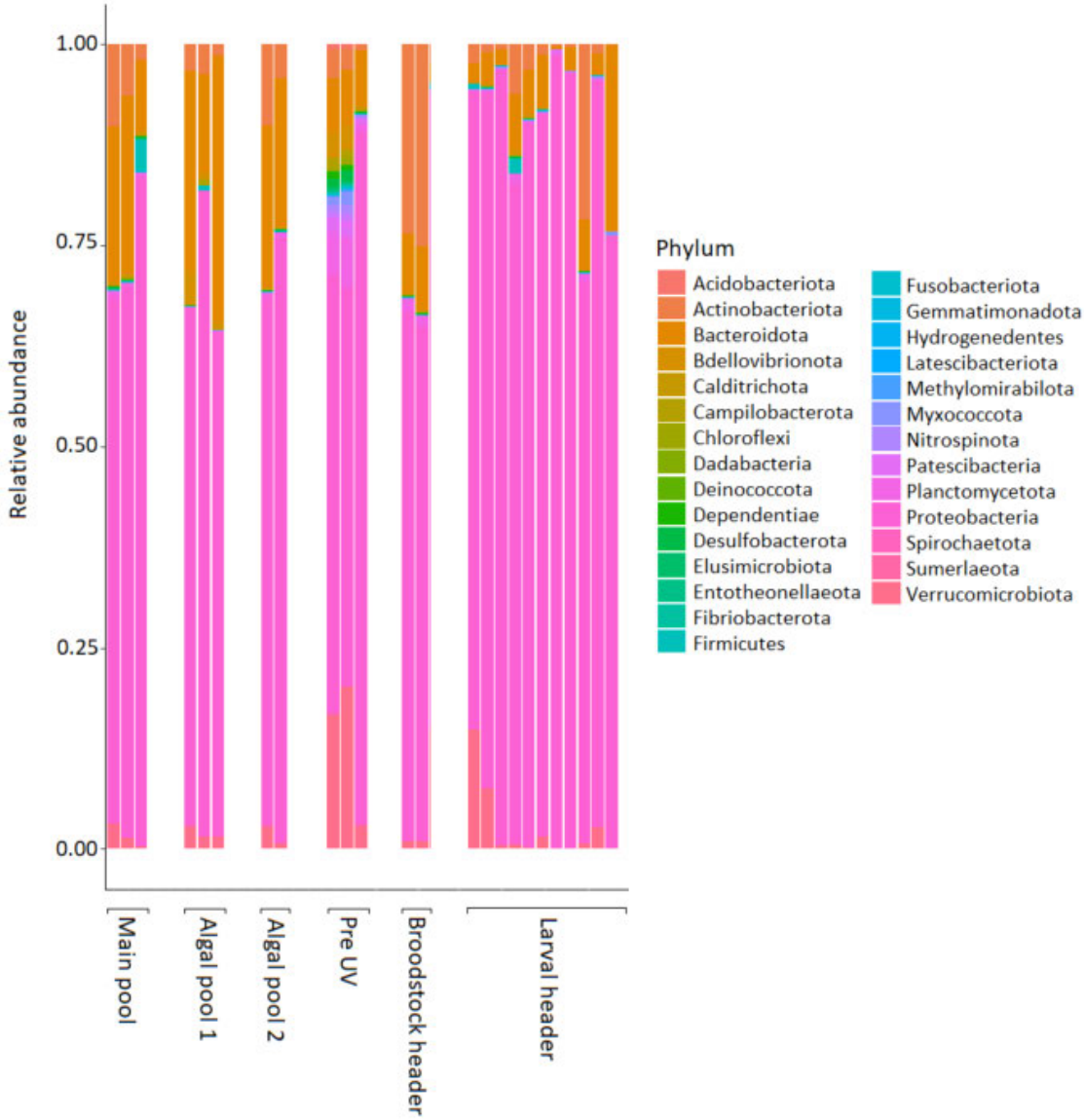


Figure 41: Relative abundance of the 28 different bacterial phyla found within hatchery water samples collected throughout the Morecambe-Bay hatchery environment.

To explore additional possible groupings within the data, the relative abundance of these 28 different bacterial phyla found within hatchery water samples are represented again in heatmap format with Euclidean clustering applied to each row and column (Figure 42). Samples collected pre-UV treatment have a higher relative abundance of most of the bacterial phyla shown in comparison to the overall mean. Samples collected from the larval header tank and broodstock header tank appear in two clear groupings. There is little variation between samples collected from these two different locations. Samples collected from larval header tanks had a higher relative abundance of Protobacteria in comparison to the overall mean value for this phylum. Samples collected from the broodstock larval tank however had a higher relative abundance of Actinobacteriota in comparison to the overall mean value for this phylum. It should be noted that the compositional differences between samples types described in Figure 41 and 42 are descriptive results, where conclusions on statistical significances cannot be given.

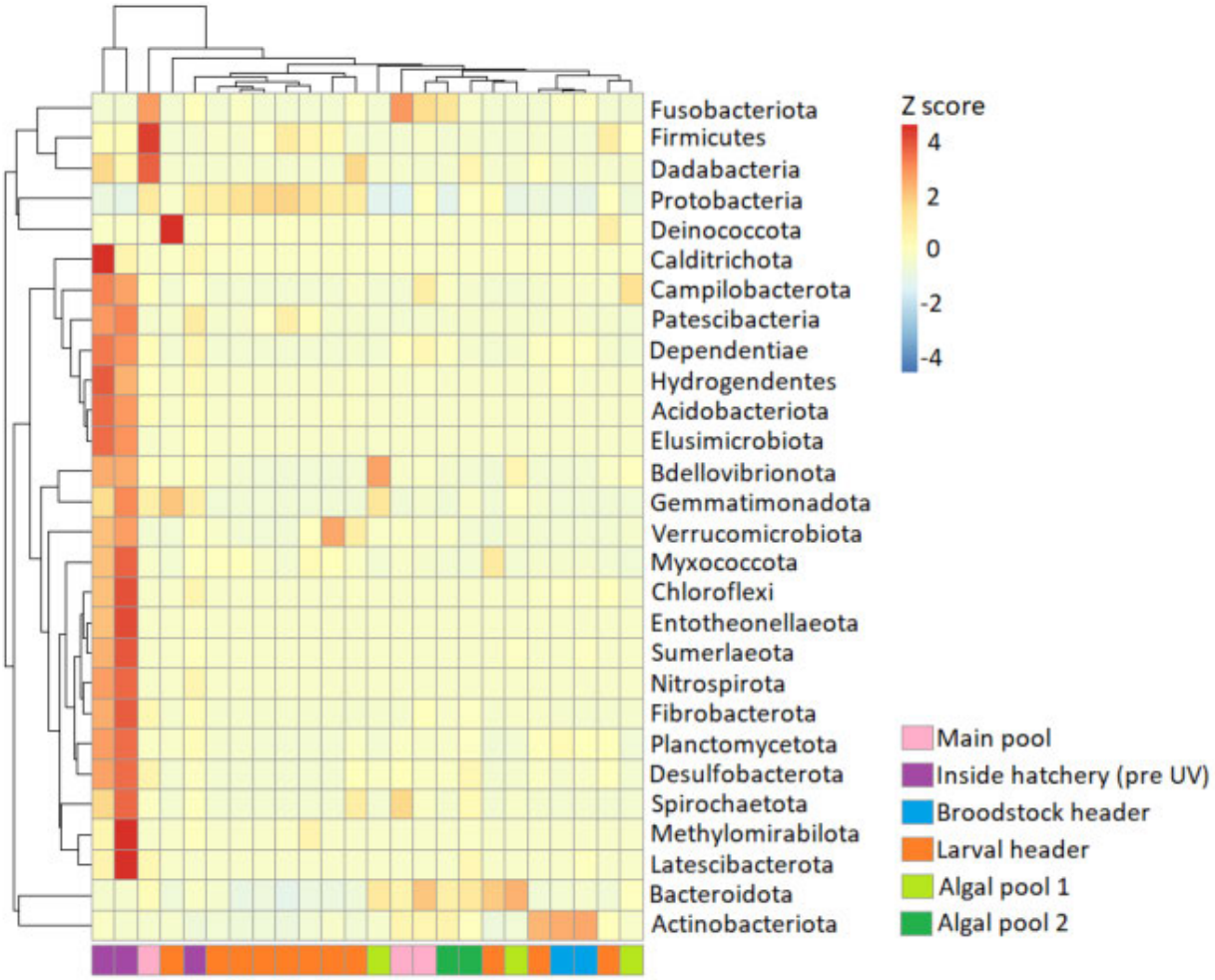


Figure 42: The relative abundance of the 28 bacterial phyla found within hatchery water samples collected throughout the Morecambe-Bay larval rearing process. Relative abundance values are normalised for expression as Z score values and Euclidean clustering applied to each row and column. Coloured annotations indicate the location of sample collection from within the hatchery.

Seven different bacterial phyla were identified amongst samples of algae (species of *T. iso* and *Pavlova*) and *axenic* (by name) starter cultures of *T. iso* (Figure 42). Fewer bacterial phyla were identified within axenic starter cultures, which were composed of either Protobacteria, Bacteroidota and Actinobacteriota, or Protobacteria, Bacteroidota only. In algal cultures of greater volumes (400 L), a greater bacterial diversity was seen. Whilst Protobacteria was still the most dominant phyla, Desulfobacterota, Firmicutes, Myxococcota and Planctomycetota phyla were also recorded. Little variation was seen between algae of different species, with

the exception of one *Pavlova* sample having a higher relative abundance of Myxococcota. Similarly, little variation was seen within algal samples collected at different time points, although *T. iso* collected on 09/06/2022 had a higher abundance of Bacteroidota than the same cultures collected on 24/05/2022 (Figure 43).

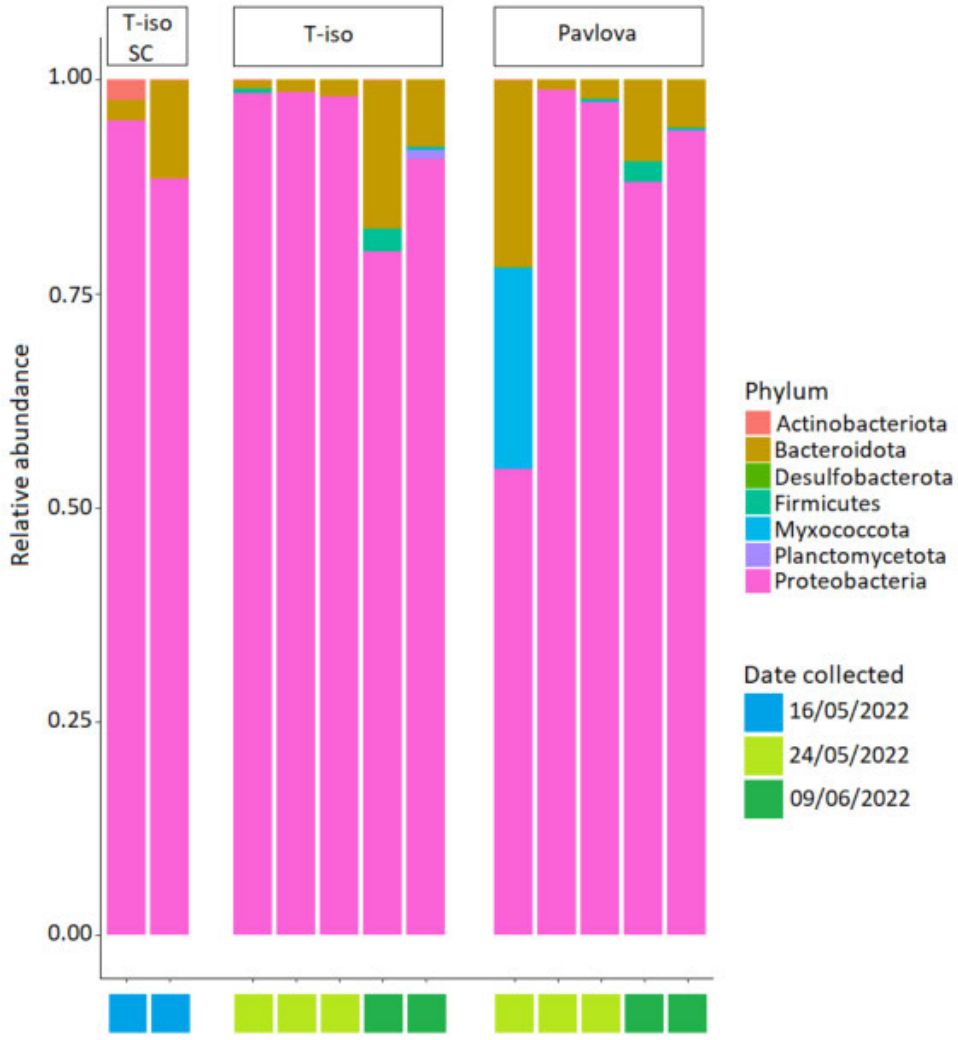


Figure 43: Relative abundance profiles of the seven different bacterial phyla found within samples taken from 400 l cultures of algae and axenic starter cultures at Morecambe Bay hatchery. Samples taken from 400 l culture bags include species of *Pavlova* and *Tisochrysis lutea (T. iso),* collected on 24/05/2022 and 09/06/2022. Samples of axenic *T. iso* starter cultures (SC) were sampled on 16/05/2022 at the beginning of the spawning event.

The seven bacterial phyla found within algal samples are shown again in a heatmap, where relative abundance levels are expressed at Z- score numbers and Euclidean clustering is applied to each row and column (see Figure 44). No clear clustering is seen, although, one of the axenic starter cultures showed a higher relative abundance of Myxococcota in comparison to the overall mean of that phyla.

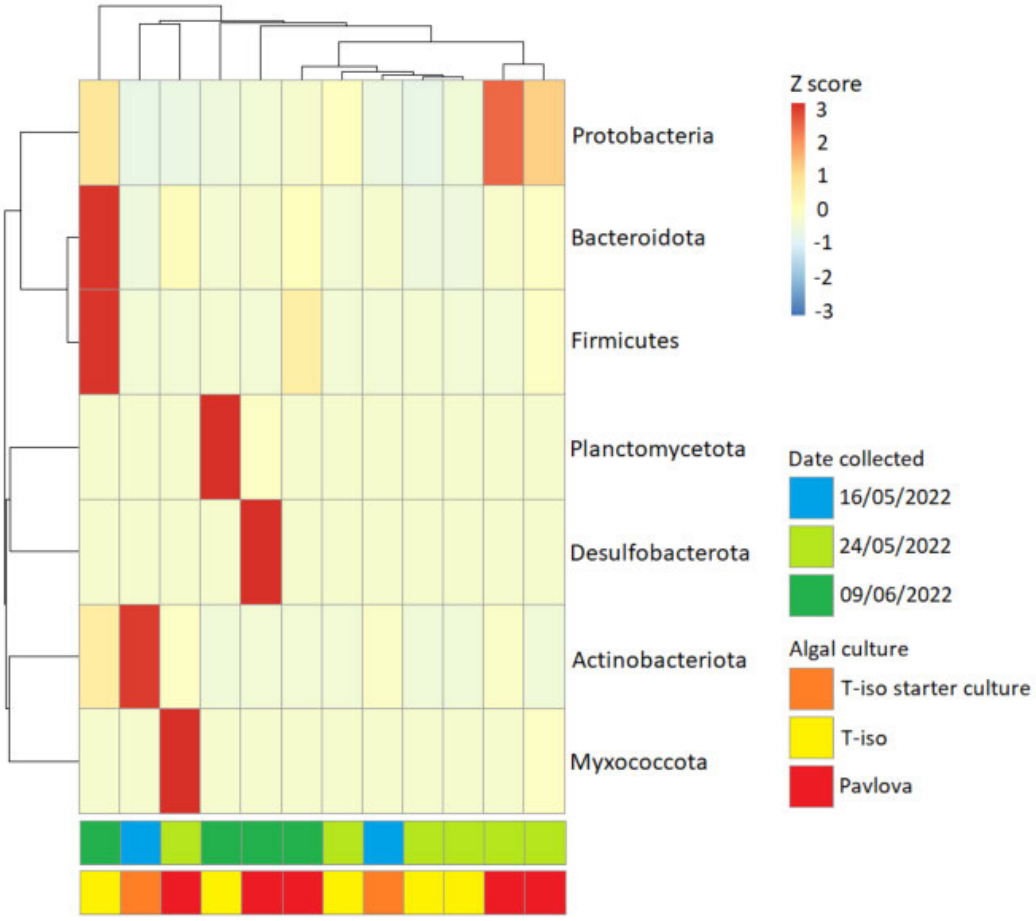
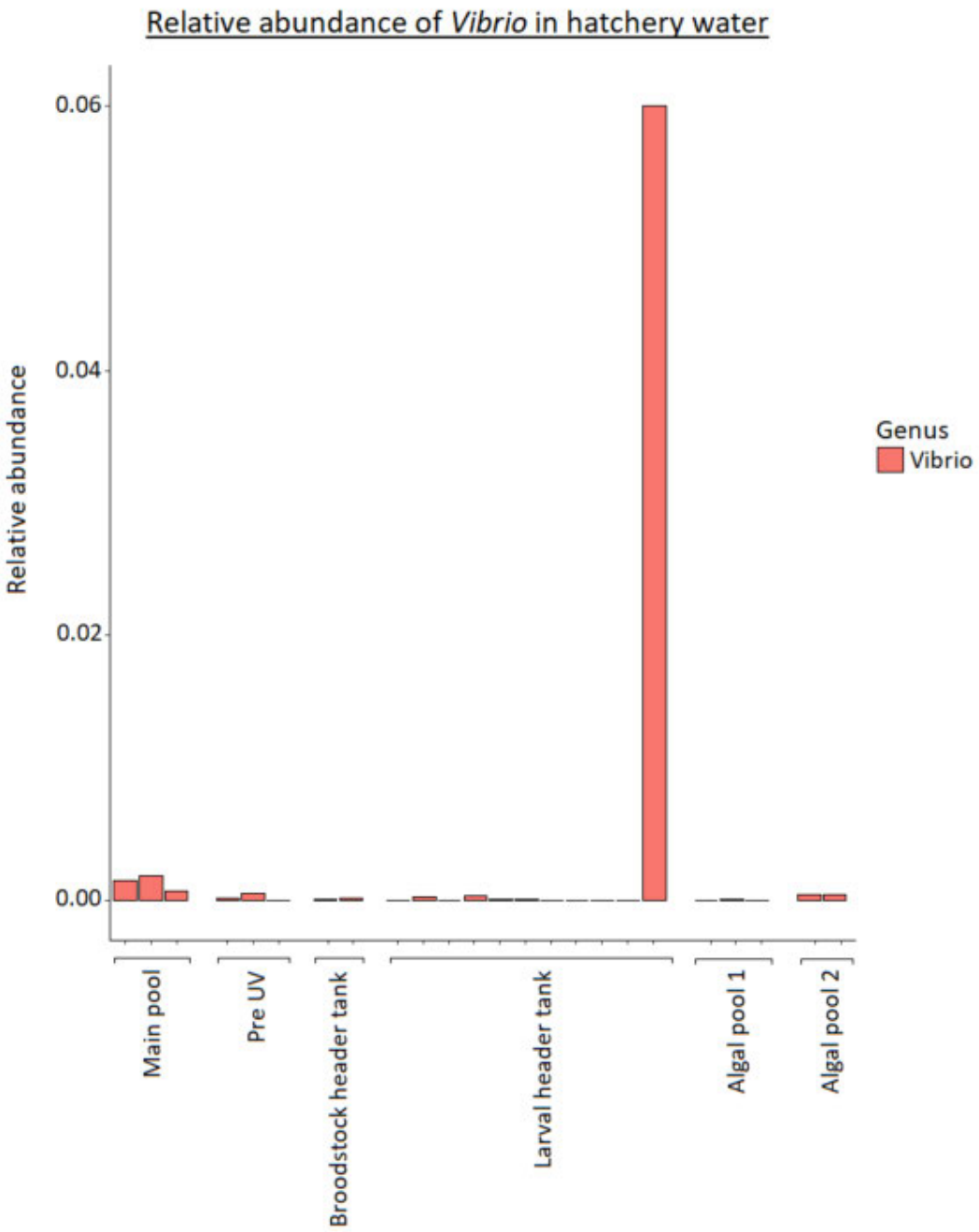
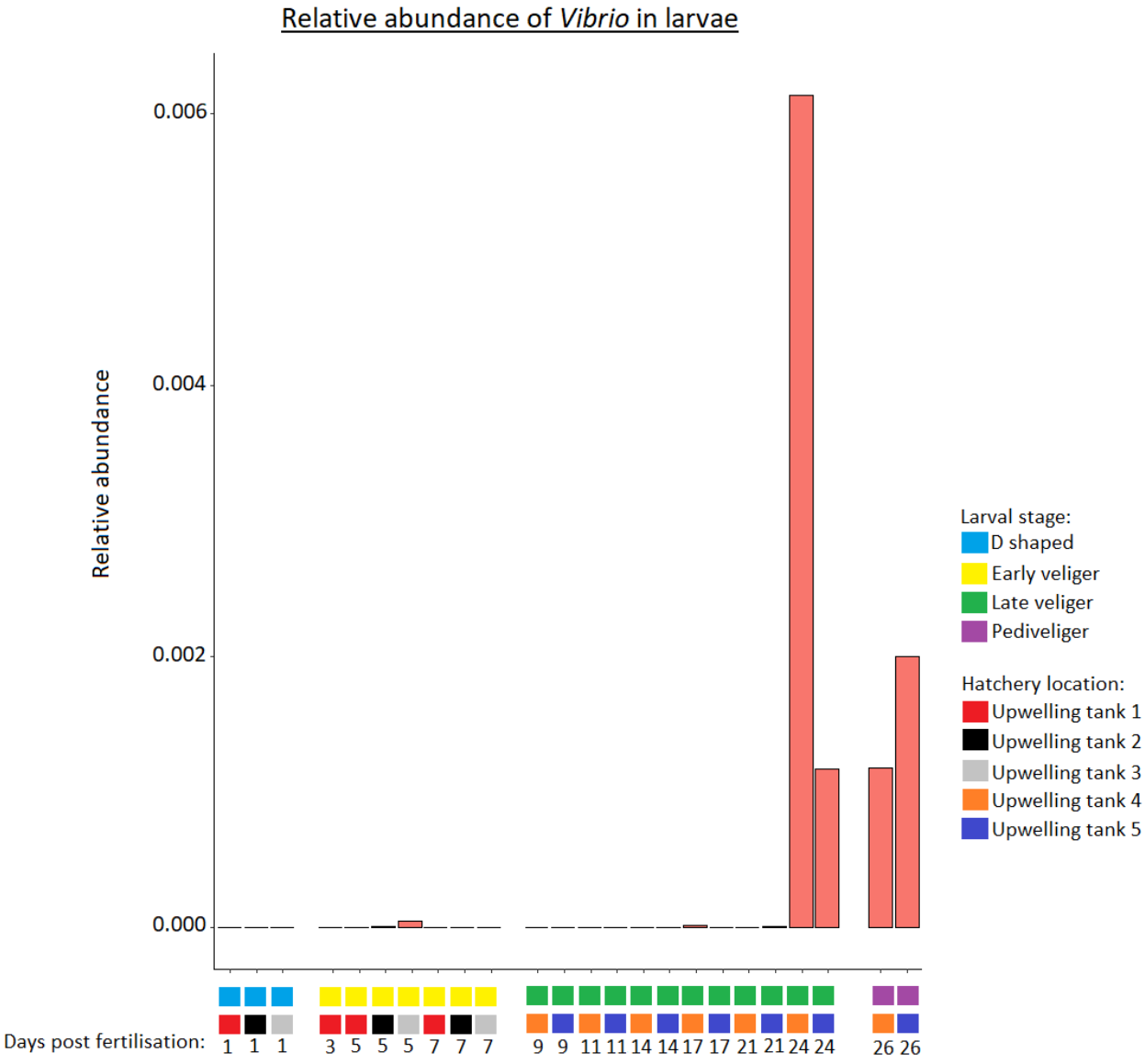


Figure 44: Heatmap showing relative abundance data of the seven different bacterial phyla found within algal samples collected from Morecambe Bay. Abundance values have been normalised for expression as Z score values and Euclidean clustering applied to each row and column. Column annotations indicate the algal species, collection date and where the sample was taken from an axenic starter culture or 400 L culture.

One OTU belonging to the *Vibrio* genus was identified in samples collected during this spawning event. Figure 45 shows the relative abundance of this *Vibrio* OTU within samples of hatchery water (a), larval samples (b) and larval culture water (c). *Vibrio* was not detected within samples of algae or axenic starter cultures.




Relative abundance of *Vibrio* in larvae
Relative abundance
0.006
0.004
0.002
0.000
Days post fertilisation:
1 1 1 3 5 5 5 7 7 7 9 9 11 11 14 14 17 17 21 21 24 24 26 26
Larval stage:
D shaped
Early veliger
Late veliger
Pediveliger
Hatchery location:
Upwelling tank 1
Upwelling tank 2
Upwelling tank 3
Upwelling tank 4
Upwelling tank 5

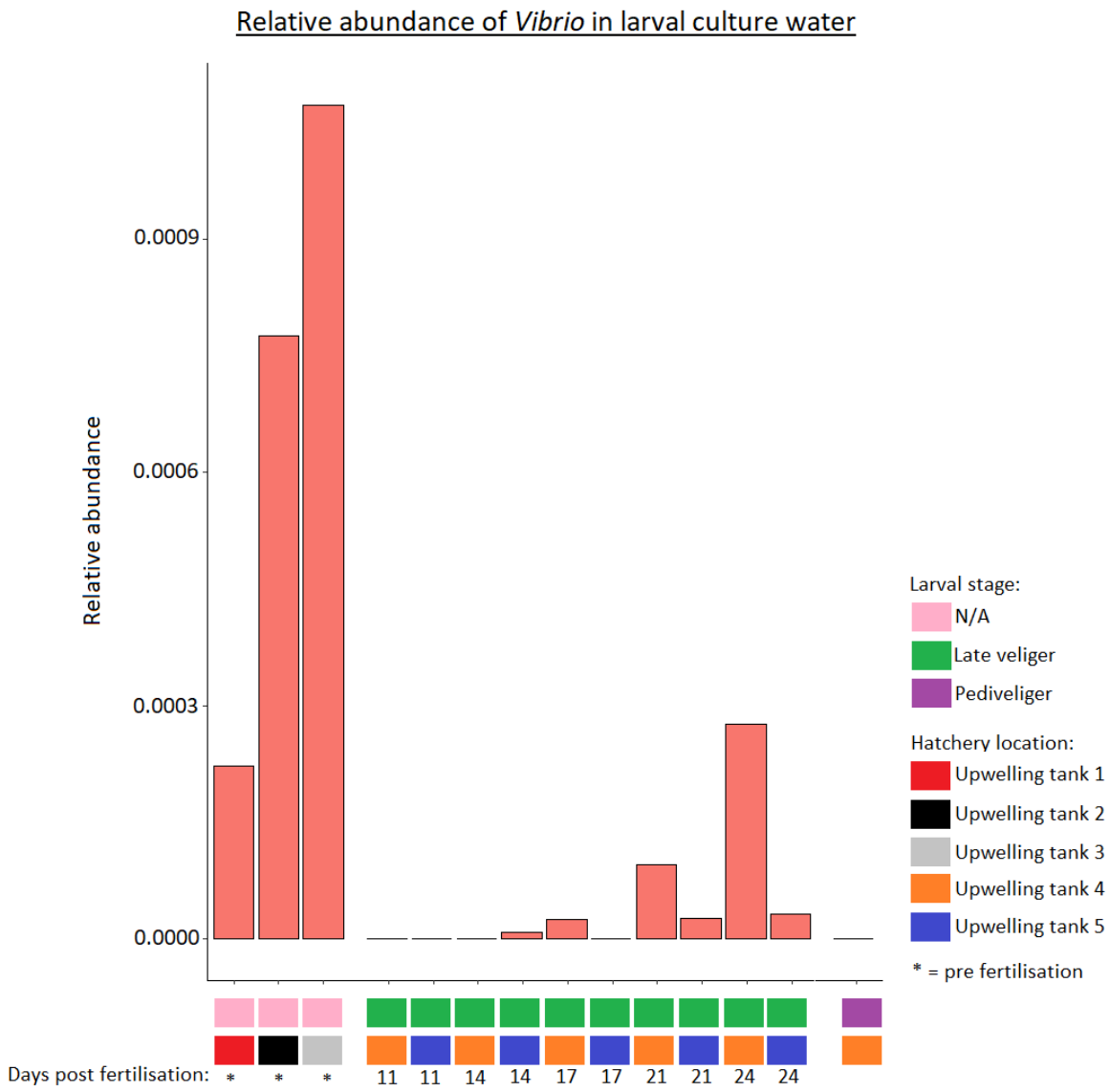


Figure 45: Relative abundance profiles of *Vibrio* bacteria found within **(a)** hatchery water samples **(b)** larval samples (grouped according to the developmental stage and location of collection). D-shaped larvae are defined as those collected between 12-24 hours post fertilisation. Early and late veliger larvae were collected between 3-7 days and 9-24 days post fertilisation respectively. Pediveliger larvae were collected at 26 days post fertilisation. **(c)** larval culture water samples (grouped according to the developmental stage of larvae location of collection). Larval culture water samples annotated as 'NA' were collected prior to the addition of larvae to upwelling tanks.

* * *

Discussion:

This chapter provides broad scale microbial data from standard oyster production runs in industrial oyster hatcheries – an environment rarely investigated within bivalve microbiome studies. Conducting research within these settings provides both advantages and limitations to the dataset presented. Since working oyster hatcheries are busy, working environments, practical and logistical difficulties resulted in sample sets sometimes not being fully comprehensive. For example, samples of larval culture water during the start of larval rearing were not collected from Morecambe Bay. This in turn provides limitations within the data analysis of aim two, where the microbiome of early oyster larvae cannot be compared to that of their surrounding water.

During commercial production, larval tanks were also merged at different time points (Figures 11 and 19). Whilst this practice is beneficial to production in terms of maintaining consistent stocking densities and reducing overhead costs, it resulted in limitations within the data collection of this chapter. For example, the merging of batches prevented the continuous sampling from the same larval tank and tracking of a single population over time. This obscures any microbial signatures which may have been specific to the developmental stage or environmental conditions of that larval batch, ultimately reducing the ability to track biologically meaningful microbial trends. This is likely to complicate the development of future microbiome-based interventions, which may be targeted to specific larval developmental stages.

Despite such limitations, these hatchery locations allowed for the collection of a unique data set which fills a knowledge gap within the current literature. In comparison to the majority of past research, which has conducted microbiome studies in more sterile aquaculture facilities, the data presented here is more representative of commercial oyster cultivation. This is advantageous in having more direct applications to the improvement of larval rearing and aquaculture generally.

Aim one: To identify core larval taxa and how these change throughout the rearing process.

Core larval taxa were reported, being defined as the collection of OTUs which were present in all larval samples. Similar numbers of core taxa were reported from each hatchery, with 41 being present within the 24 larval samples from Morecambe Bay, and 42 within the 19 larval samples from Portsmouth (see Figures 20 and 35). Although present at different relative abundances, these core larval taxa were present within all of the larval stages investigated. The presence of core bacterial taxa which persist throughout different stages has been previously reported in studies of *Saccostrea glomerata* (Sydney rock oyster) in a research-orientated environment (Unzueta-Martínez et al, 2022b). Of the core taxa identified by Unzueta-Martínez et al, 2022b, three (belonging to Alphaproteobacter, Bacteroidota and Gammaproteobacteria) could also be traced back to being present within the gametes - suggesting some vertical transmission of microbes. Unfortunately, although gamete samples from the strip spawning at Morecambe Bay were collected, bacterial DNA was not sufficiently amplified and so a similar comparison cannot be made. This lack of bacterial DNA amplification may possibly have been caused by PCR inhibitions unique to this sample type.

Core larval taxa from Morecambe Bay are annotated to show two main clustering's of OTUs, with pediveliger and D-shaped larvae belonging to different groupings (Figure 35). With the exception of larvae collected at a maximum of 12 hours post larval release, larvae of the later developmental stages at Portsmouth also showed different clustering to early developmental stages (Figure 20). This data (Figures 20 and 35), describes changes to the core microbiome as larvae progress throughout the different developmental stages – an important prerequisite for the development of microbiome-based intervention strategies.

OTUs belonging to a total of 11 bacterial genera (or families) were common to core larval taxa at Morecambe Bay and Portsmouth: Rhodobacteraceae, *Allorhizobium-Neorhizobiom-Pararhizobiom-Rhizobiom*, Brevundimonas, Rhizobiales, *Mesorhizobium*, *Acinetobacter*, *Microbacterium*, Alcaligenaceae, *Achromobacter*, *Paracoccus* and *Tateyamaria*. Notably, Rhodobacteraceae is a diverse bacterial family common to the marine environment, and has been previously reported within the larval microbiomes of *C. gigas, Crassostrea virginica, O. edulis* and *Saccostrea glomerata* at varying developmental stages (Arfken et al, 2021; Asmani et al, 2016; Cho et al, 2024, Unzueta-Martínez et al, 2022b; Chapter two of this thesis). Being

common to core larval microbiomes within two different oyster species and hatcheries, these OTUs are most likely to have important functional roles within the host. Understanding the functional role of such key taxa would aid in the understanding of larval health and development – information which could be of great value to the hatchery processes. Future studies may therefore benefit from utilising bioinformatic tools such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), to report predictive functional profiles of the microbiomes reported.

OTUs belonging to a total of 11 bacterial genera (or families) were common to core larval taxa at Morecambe Bay and Portsmouth: Rhodobacteraceae, *Allorhizobium-Neorhizobiom-Pararhizobiom-Rhizobiom*, Brevundimonas, Rhizobiales, *Mesorhizobium*, *Acinetobacter*, *Microbacterium*, Alcaligenaceae, *Achromobacter*, *Paracoccus* and *Tateyamaria*. Being bacterial taxa common to all larval stages at both hatcheries (and two different species), they may therefore play important functional roles within larval development. Again, future research would benefit greatly from investigating possible functional roles of these genera. In comparison to existing literature, these genera have not previously been reported within the core oyster larval microbiome with previous studies of *C. gigas* and *S. glomerata* (Cram et al, 2024; Unzueta-Martínez et al, 2022b). Differences in core larval taxa may be attributed to a wide range of variables including oyster species, season, spawning technique, hatchery type or location. It should however be noted that these bacterial genera were however also identified in seawater samples, meaning that their presence may be explained by the larval environment rather than being of any particular biological importance. Future research may therefore also aim to better understand this relationship in order to fully report on the functionalities of these genera.

An OTU belonging to the *Vibrio* genus was present within the core microbiome of larval samples collected from Portsmouth, but not Morecambe Bay. Species of this genus are very common in water (Baker-Austin et al, 2018; Pruzzo et al, 2005) and whilst they are also known to cause disease mortality events to larval stocks, larvae reared at Portsmouth were observed to be healthy, mobile and unaffected by disease outbreaks. In previous studies investigating failed larval productions, *Vibrio* were detected in some but not all crashed larval populations (Cram et al, 2024). Whilst the majority of literature is focused upon the effects of pathogenic

Vibrio sp., the presence of this genus does not necessarily indicate failing larval stocks or a problem with the hatchery system.

In both hatcheries, fewer OTUs are over-represented with larvae of the later developmental stages than the earlier developmental stages (Figures 20 and 35). This reduction in species richness over time may suggest that the core larval microbiome stabilises over time and is less likely to be influenced by external environmental influencing factors. A similar hypothesis is made by Cho et al, 2024 after reporting that the variability in diversity and richness of the core taxa in *C. gigas* spat decreased with age. In comparison, the overall larval microbiomes (not core taxa only), the species richness of larvae collected from Portsmouth also decreased as development progressed (Figure 24). Whilst this may again suggest evidence that later larval stages are less influenced by microbes provided from the external environment, alpha diversity was ultimately not statistically different between developmental stages (Observed p=0.7901 and Chao1 p = 0.965); (Figure 24). Conversely, the overall microbiome of larvae collected from Morecambe Bay increased in species richness overtime, although statistically this result was again not significant (Observed p = 0.6085 and Chao 1 = 0.7793); (Figure 37). Unfortunately, sequencing such a broad range of sample types across the whole spawning period resulted in few larval replicates from each time point being able to be included in the final data set. These small larval sample sizes from each time point are acknowledged, with possible impacts to that statistical power of the Kruskal-Wallis test used. Future studies conducted within commercial hatcheries are therefore advised to increase sample sizes, or include biological replication to increase the power of downstream statistical testing.

Core microbial taxa from these different spawning events have been reported and provide important information about the bacterial groups which are most likely to influence larval health and development. In agreement with existing literature, changes in core taxa were more closely associated with the progression of larval development (Unzueta-Martínez et al, 2022b) than hatchery location (tank number from which the sample was collected from). Future research would significantly benefit from investigating functional roles of core taxa – especially those belonging to *Rhodobacteraceae,* since this family is reported consistently within different oyster species and environments (Arfken et al, Asmani et al, 2016; Cho et al, 2024, Unzueta-Martínez et al, 2022b).

* * *

Aim two: Compare bacterial profiles of larvae to those of their hatchery environment.

NMDS analysis compared microbial profiles of larval samples with microbial profiles of other sample types (hatchery water, larval culture water, algae, algal starter cultures and biofilm); (Figures 21 and 36). Within both hatcheries, sample type drove a greater percentage of microbial variation within the dataset than the stage of larvae development (r^2 = 0.354 at Portsmouth and r^2 = 0.323 at Morecambe Bay). Considering that *O. edulis* larvae spend the beginning of their life cycle within the pallial cavity before being released into the external water, it can be expected that the microbiome of the cavity will influence that of larvae collected soon after larval release. Indeed, data points representing pallial cavity water samples were grouped most closely with larvae released between 13 hours and 6 days post larval release at Portsmouth (Figure 21). Larval samples collected from Morecambe Bay were grouped most closely with larval culture water samples collected during the later stages of larval development, and also biofilm (Figure 36). The formation of biofilm in aquaculture settings is initiated by the colonisation of free-floating bacteria from within the water (Cai and Arias, 2017), meaning that the biofilm microbiota is likely influenced by both larvae and algae. Similarities between the microbiome of *C. virginica* larvae and their tank biofilms have been previously reported, with both having a lower diversity index then the larval culture water. This may suggest niche selection of larval and biofilm colonisers (Stevick et al, 2019). The larval microbiome is therefore most likely influenced by both water and algal microbiomes, which together selectively colonise biofilm layers in tanks.

As free swimming, filter feeding animals it can be expected that the microbiome of oyster larvae have a close association with that of their surrounding water. Microbial comparisons between these two sample types have been previously reported as distinct from one another. Studies of *S. glomerata* larvae are shown to have a higher species richness than their surrounding environment, with 385 ASV (amplicon associated variants) being present in larvae but not water (Unzueta-Martínez et al, 2022b). In hatchery production of *C. virginica,* larvae were found to have a higher relative abundance of *Gammaproteobacteria* than the surrounding water (Arfken et al, 2021). Such studies provide important information needed

to determine the source of the larval microbiome. The samples collected throughout this chapter allows for the similar comparisons, but during larval production on a commercial scale.

Within the Portsmouth data set, species richness levels were not statistically different between samples of larvae or larval culture water (Figure 22). Microbial differences between these sample types are therefore driven by abundance levels of certain taxa – a similar finding as reported by Arfken et al, 2021. Species richness levels within both of these sample types did however decrease as larval developmental stages progressed (Figure 24, Figure 25). This reduction in species diversity could again suggest stabilisation of the larval microbiome in favour of taxa which are more beneficial to larvae at that developmental stage. Comparisons between species richness levels of larval and larval culture water samples at Morecambe Bay are compromised by a lack of larval culture water samples collected at the start of the spawning event. Despite this missing time point, a reduction in species diversity within larval culture water samples overtime was again reported (Figure 38). This trend was however not replicated within larvae samples, where species richness increased as developmental stages progressed (Figure 37). Although an increasing species richness has been previously correlated with larval mortality of *C. gigas*, this was not observed at Morecambe Bay (Vignier et al, 2021). Although not driven by differences in species richness, microbial differences between these sample types at both hatcheries suggest that the larval microbiome is not merely a reflection of the environment. This is not to say that environmental parameters do not affect the larval microbiome, but that it also be selectively shaped by the host.

Differential abundance analysis was used to compare changes in the microbial profiles of larvae over time at Portsmouth (Figure 28). The majority of OTUs identified within this analysis were more significantly more abundant in larvae between 7 – 10 days post larval release than 13 hours – 6 days post larval release. This may provide evidence of the named taxa becoming more established within the larval microbiome to carry out functional roles needed throughout development.

* * *

Aim three: Investigate how hatchery processes specific to these locations including water filtration and algal cultivation methods influence microbial communities.

Practices of selecting and merging together the largest larvae from different upwelling tanks allows hatcheries to carry forward the most suitable larvae and promote uniform growth across batches (Figures 11 and 18). A key consequence of this within the sample collection of this study was that singular larval populations could not be continually tracked. This process may mean that microbial signatures distinct to each larval bin would be lost upon merging, as microbial taxa are exchanged between larvae from different batches. Larvae at Morecambe Bay were produced from the same broodstock oysters. Therefore, microbial signatures distinct to larval bins at this location would most likely have been driven by slight inconsistencies in environmental conditions such as water parameters or feeding regimens. Analysis of core and general larval taxa at Morecambe Bay did however not display any clear groupings of taxa associated with certain larval upwellings prior to their merging (larval upwelling tanks one, two and three); (Figures 18, 35 and 40).

Larvae at Portsmouth were obtained from two sets of broodstock oysters (two different natural spawning events). Any microbial signatures distinct to different larval trays (Figure 11) may therefore either have been detected as a result of inconsistencies in environmental conditions or differences in gamete microbiota. However, analysis of core taxa and general larvae taxa at Portsmouth also showed no clear groupings of taxa associated with certain larval trays or upwellings tanks (Figures 11, 20 and 27). Although it may be expected that the merging of tanks would result in the sharing of taxa between previously separated larvae, this hypothesis is difficult to conclude when tank-specific microbial signatures were not detected prior to the merging of tanks at either hatchery. The inability to continuously track the same larval batch throughout production is an important consequence of the hatchery process and key consequence of studying the larval microbiome in a commercial versus research-focused setting.

Water filtration allows for the improvement of water quality and removal of potentially harmful microorganisms – making it a vital part of oyster hatchery production. Collecting microbial data from the hatchery environment therefore provides an important opportunity to inform hatcheries about the efficiency of their specific water filtration methods. This is of

a direct advantage, allowing the hatchery teams to improve practices quickly if needed. This could reduce the likelihood of a disease outbreak event within larval cultures, improving seedstock output and economic gain for the hatchery.

The intake water filtration practices at both locations have clear implications on the hatchery water microbiomes. At Portsmouth, OTUs belonging to Proteobacteria, Bacteroidota and Acidobacteritoa were the most relatively abundant within hatchery water samples (Figure 29). These are phyla also found to be dominant across other hatcheries regardless of location or season (Vignier et al, 2021). Water samples collected from header and broodstock tanks had lower relative abundances of Verrucomicrobiota, Nitrospinota, Dependentiae, Desulfobacterota, Elusimicrobiota and Fibrobacterota compared to those collected throughout different filtration stages (Figures 29 and 30). This suggests that filtration methods at Portsmouth were successful in reducing microbial diversity of the water, most likely whilst also reducing overall numbers of microbes. It should be noted that relative abundance data does not reflect the overall bacterial load of the sample. To confirm that the filtration process was successful in reducing overall bacterial load, future studies may therefore benefit from incorporating techniques such as qPCR or calculation of the culturable bacterial load with CFU/ml data.

Differential abundance analysis (Figure 31) identified OTUs which were significantly less abundant in water samples from header tanks one and two in comparison to those taken from the harbour. This supports further supports the finding that water filtration methods at Portsmouth were successful in reducing bacterial load. However, some taxa displayed very high $\log_2$ fold change values, which may be artificially inflated due to small sample sizes and low read counts. Such conditions are known to introduce statistical noise and reduce the reliability of differential abundance estimates. Therefore, caution is warranted when interpreting these extreme values. In this analysis, only OTUs from families of Omnitrophaceae, Rubinisphaeraceae, Bacteriovoracaceae (Figure 31a), and Bdellovibrionaceae, Rubinisphaeraceae and Omnitrophaceae (Figure 31b) are considered biologically meaningful. Future work should prioritise increased replication to improve the statistical power and robustness of differential abundance findings.

At Morecambe Bay, a higher relative abundance of most phyla (in comparison to their overall mean) in pre-UV samples compared to all other hatchery water sample types was seen (Figure 42). Again, this shows that the UV treatment effectively reduced microbial diversity. A reduction in microbial diversity is possibly of benefit of larval cultures, with species richness being previously positively correlated with larval mortalities (Vignier et al, 2021). Again, it is important to note that this not a reflection of overall bacterial load, and that water samples taken pre-UV had already passed through pasteurisation, and cartridge filters to reduce the overall bacterial abundance. The use of pasteurisation is practiced at Morecambe Bay, but not at Portsmouth. Possible microbial implications of pasteurisation can be analysed by comparing water samples collected from the main pool of Morecambe Bay to those collected pre-UV treatment. When comparing these sample types, little difference is seen in the relative abundance of *Vibrio* (Figure 45a). However, water samples collected from the main pool did show a greater relative abundance of *Bacteroidota* than those collected pre-UV treatment (Figure 40). Whilst this microbial implication may be a result of pasteurisation, it cannot be ruled out this this is also an effect of the use of cartridge filters.

Algal cultivation and feeding are necessary practices for hatcheries and have the potential to influence the larval microbiome. Algal cultures are a potential source of both transient and core microbial taxa to larvae, as well as pathogenic bacteria. A link between algal and larval microbiomes has been previously established, finding a positive association between larvae fed with compromised algae (with an elevated pH) and impaired larval fitness (deformities, slowed development, reduced swimming and feeding); (Vignier et al, 2021). More specifically, genera of *Halomonas* and *Marinomonas* were associated with impaired larval performance, whilst *Vibrio* was associated with overfeeding (Vignier et al, 2021). This finding highlights how maintenance of healthy larval cultures are vital for successful larval production runs. Whilst similar experiments would be useful to recreate during commercial production, the deliberate compromising of algal and larval cultures would come at an economic loss to the hatchery.

The majority of algal samples (and larvae samples) at both hatcheries were comprised of Protobacteria and Bacteroidetes (Figures 32, 33, 43 and 44). This is a finding also shared by algal analysis within more sterile research environments (Vignier et al, 2021). Arfken et al, 2021 reports that Rhodobacteraceae are abundant in phytoplankton cultures, and that therefore algal feed may be the source of this key taxa to larvae. Rhodobacteraceae (of the

Phylum Pseudomonadota) was however not detected in algal samples from either hatchery (Figures 32 and 43). Bacterial taxa present in *C. gigas* larvae but absent from algal cultures have been previously reported, although these did not include those belonging to Rhodobacteraceae (Vignier et al, 2021). Similarly, an OTU belonging to the *Vibrio* genera was identified as a member of the core larval taxa at Morecambe Bay (Figure 35). This genus was however not detected within algal samples collected from this hatchery (Figure 43, Figure 44). Therefore, although algal are larval microbiomes are closely associated (Vignier et al, 2021), the presence of bacterial taxa within algae does not guarantee their transfer to the larvae. This is supportive of the idea that the larval microbiome is somewhat distinct to their external surroundings and apply some level of selective control over their own microbiome.

* * *

Conclusion and future recommendations:

This chapter provides in-depth data to investigate the microbiome of oyster larvae and their hatchery environment. A broad range of sample types were analysed, which allowed for a comprehensive overview of the hatchery microbiome, incorporating two different locations, oyster species and spawning types.

Research of the larval microbiome and their hatchery environment has important potential to better inform microbial manipulation strategies within commercial production. Probiotic treatments are arguably the fastest developing method of microbial manipulation within shellfish aquaculture, most frequently administered through the water or feed (Sumon et al, 2022). Although the microbiome of larvae were found to be distinct to that of algae or their surrounding water, these sample types still have potential to influence the larval microbiome. It could be suggested the adding probiotic treatments to algae instead of water would be beneficial in directly reaching the digestive tract of larvae and therefore having a more direct effect. Administering probiotics to broodstock oysters may be an alternative method of probiotic administration. This can be suggested after reporting a close association between the larval and pallial cavity water microbiomes at Portsmouth, and past studies showing both

horizontal and vertical transmission of the microbiome (Unzueta-Martínez et al, 2022b). Although the efficacy of this method is yet to be tested, it would be advantageous with far lower quantities of probiotics being needed to treat broodstock than larval batches.

Conducting this study in a commercial environment provides data which is lacking within the current literature, yet directly applicable to the hatchery environment and improvement of oyster aquaculture. Although not within the scope of this study, these data could be further improved by incorporating more hatchery locations, biological replication, and sequencing of samples collected more regularly throughout larval rearing. For example, similar sample sets could also be collected from UK locations such as Guernsey Sea Farms or the Orkney Shellfish Hatchery (OSH), to further the understanding of the aims investigated here. Future studies would also benefit from incorporating techniques such as qPCR and bacterial plating on general marine and selective agar to collect bacterial loads and culturable bacterial loads from similar sample sets. This may aid in determining the functional roles of bacteria within oyster larvae and their environment – a significant knowledge gap within the current literature.

References:

Adams, S. L., Salinas-Flores, L., & Lim, M. H. (2013). Diet conditioning of pacific oyster, *Crassostrea gigas*, broodstock to improve oocyte cryopreservation success. *Journal of Shellfish Research*, 32(2), 391–399. https://doi.org/10.2983/035.032.0219

Allen Jr, S.K. and Bushek, D. (1992). Large-scale production of triploid oysters, *Crassostrea virginica* (Gmelin), using "stripped" gametes. *Aquaculture*, 103(3-4), 241-251.

Arfken, A., Song, B., Allen, S. K., & Carnegie, R. B. (2021). Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. *Aquaculture*, 531. https://doi.org/10.1016/j.aquaculture.2020.735955

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*, 464, 434–444. https://doi.org/10.1016/j.aquaculture.2016.07.020

Baker-Austin, C., Oliver, J.D., Alam, M., Ali, A., Waldor, M.K., Qadri, F. and Martinez-Urtaza, J. (2018). *Vibrio* spp. infections. *Nature Reviews Disease Primers*, 4(1), 1-19.

Barbosa Solomieu, V., Renault, T., & Travers, M. A. (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas. Journal of Invertebrate Pathology*, 131, 2–10. https://doi.org/10.1016/j.jip.2015.07.011

Botta, R., Asche, F., Borsum, J. S., & Camp, E. V. (2020). A review of global oyster aquaculture production and consumption. *Marine Policy*, 117. https://doi.org/10.1016/j.marpol.2020.103952

Brown, M. R., & Blackburn, S. I. (2013). Live microalgae as feeds in aquaculture hatcheries. *Advances in aquaculture hatchery technology*, 117-158.

Cai, W. and Arias, C.R., 2017. Biofilm formation on aquaculture substrates by selected bacterial fish pathogens. *Journal of Aquatic Animal Health*, 29(2), 95-104.

Chen, C. Y., Chen, P. C., Weng, F. C. H., Shaw, G. T. W., & Wang, D. (2017). Habitat and indigenous gut microbes contribute to the plasticity of gut microbiome in oriental river prawn during rapid environmental change. *PLoS ONE*, 12(7). https://doi.org/10.1371/journal.pone.0181427

Cho, A., Finke, J. F., Zhong, K. X., Chan, A. M., Saunders, R., Schulze, A., Warne, S., Miller, K. M., & Suttle, C. A. (2024). The core microbiome of cultured Pacific oyster spat is affected by age but not mortality. *Microbiology Spectrum*. https://doi.org/10.1128/spectrum.00031-24

Cole, H. A. (1951). The British oyster industry and its problems. Rapports and Proces-Verbaux des Reunions. *Conseil Permanent International pour l'Exploration de la Mer*, 128, 7-17.

Colsoul, B., Boudry, P., Pérez-Parallé, M. L., Bratoš Cetinić, A., Hugh-Jones, T., Arzul, I., Mérou, N., Wegner, K. M., Peter, C., Merk, V., & Pogoda, B. (2021). Sustainable large-scale

production of European flat oyster (*Ostrea edulis*) seed for ecological restoration and aquaculture: a review. In *Reviews in Aquaculture*, 13 (3), 1423–1468. John Wiley and Sons Inc. https://doi.org/10.1111/raq.12529

Coon, S. L., Fitt, W. K., & Bonar, D. B. (1990). Competence and delay of metamorphosis in the Pacific oyster Crassostrea gigas. *Marine Biology*, 106, 379-387.

Cram, J.A., McCarty, A.J., Willey, S.M. and Alexander, S.T. (2024). Microbial community structure variability over the development of healthy and underperforming oyster larval hatchery broods. *Frontiers in Aquaculture*, 3, 1427405.

Ford, S.E., Xu, Z. and Debrosse, G. (2001). Use of particle filtration and UV irradiation to prevent infection by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) in hatchery-reared larval and juvenile oysters. *Aquaculture*, 194(1-2), 37-49.

Helmer, L., Farrell, P., Hendy, I., Harding, S., Robertson, M., & Preston, J. (2019). Active management is required to turn the tide for depleted *Ostrea edulis* stocks from the effects of overfishing, disease and invasive species. *PeerJ*, 2019(2). https://doi.org/10.7717/peerj.6431

Humphreys, J., Herbert, R. J. H., Roberts, C., & Fletcher, S. (2014). A reappraisal of the history and economics of the Pacific oyster in Britain. *Aquaculture*, 428–429, 117–124. https://doi.org/10.1016/j.aquaculture.2014.02.034

Jorquera, M.A., Valencia, G., Eguchi, M., Katayose, M. and Riquelme, C. (2002). Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment. *Aquaculture*, 207(3-4), 213-224.

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

Kunselman, E., Minich, J. J., Horwith, M., Gilbert, J. A., & Allen, E. E. (2022). Variation in Survival and Gut Microbiome Composition of Hatchery-Grown Native Oysters at Various Locations within the Puget Sound. *Microbiology Spectrum*, 10(3). https://doi.org/10.1128/spectrum.01982-21

Lorgen-Ritchie, M., Clarkson, M., Chalmers, L., Taylor, J. F., Migaud, H., & Martin, S. A. M. (2021). A Temporally Dynamic Gut Microbiome in Atlantic Salmon During Freshwater Recirculating Aquaculture System (RAS) Production and Post-seawater Transfer. *Frontiers in Marine Science*, 8. https://doi.org/10.3389/fmars.2021.711797

Lucas, J , S., Southgate, P, C and Tucker, C, S. (2019). Aquaculture; Farming Aquatic Animals and Plants. John Wiley & Sons Ltd. Third Edition.

McCausland, M. A., Brown, M. R., Barrett, S. M., Diemar, J. A., & Heasman, M. P. (1999). Evaluation of live microalgae and microalgal pastes as supplementary food for juvenile Pacific oysters (*Crassostrea gigas*). *Aquaculture*, 174(3-4), 323-342.

Nowland, S.J., O'Connor, W.A., Elizur, A. and Southgate, P.C. (2021). Evaluating spawning induction methods for the tropical black-lip rock oyster, *Saccostrea echinata*. *Aquaculture Reports*, 20, 100676.

Pernet, F., Lupo, C., Bacher, C., & Whittington, R. J. (2016). Infectious diseases in oyster aquaculture require a new integrated approach. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1689). https://doi.org/10.1098/rstb.2015.0213

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., & Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society B*, 287(1926), 20200184.

Pimentel, Z. T., Dufault-Thompson, K., Russo, K. T., Scro, A. K., Smolowitz, R. M., Gomez-Chiarri, M., & Zhang, Y. (2021). Microbiome Analysis Reveals Diversity and Function of Mollicutes Associated with the Eastern Oyster, *Crassostrea virginica* . MSphere, 6(3). https://doi.org/10.1128/msphere.00227-21

Pohlner, M., Dlugosch, L., Wemheuer, B., Mills, H., Engelen, B., & Reese, B. K. (2019). The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. Frontiers in Microbiology, 10, 659. https://doi.org/10.3389/fmicb.2019.00659

Pruzzo, C., Huq, A., Colwell, R.R. and Donelli, G., 2005. Pathogenic *Vibrio* species in the marine and estuarine environment. *Oceans and health: pathogens in the marine environment*, 217-252.

Regan, T., Bean, T. P., Ellis, T., Davie, A., Carboni, S., Migaud, H., & Houston, R. D. (2021). Genetic improvement technologies to support the sustainable growth of UK aquaculture. *Reviews in Aquaculture*, 1–28. https://doi.org/10.1111/raq.12553

Stevick, R. J., Sohn, S., Modak, T. H., Nelson, D. R., Rowley, D. C., Tammi, K., Smolowitz, R., Lundgren, K. M., Post, A. F., & Gómez-Chiarri, M. (2019). Bacterial community dynamics in an oyster hatchery in response to probiotic treatment. *Frontiers in Microbiology*, 10, 1060, https://doi.org/10.3389/fmicb.2019.01060

Sumon, T.A., Hussain, M.A., Sumon, M.A.A., Jang, W.J., Abellan, F.G., Sharifuzzaman, S.M., Brown, C.L., Lee, E.W., Kim, C.H. and Hasan, M.T. (2022). Functionality and prophylactic role of probiotics in shellfish aquaculture. *Aquaculture Reports*, 25, 101220.

Unzueta-Martínez, A. (2022a). Determining the Composition of Resident and Transient Members of the Oyster Microbiome. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.828692

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022b). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, 4(1). https://doi.org/10.1186/s42523-022-00186-9

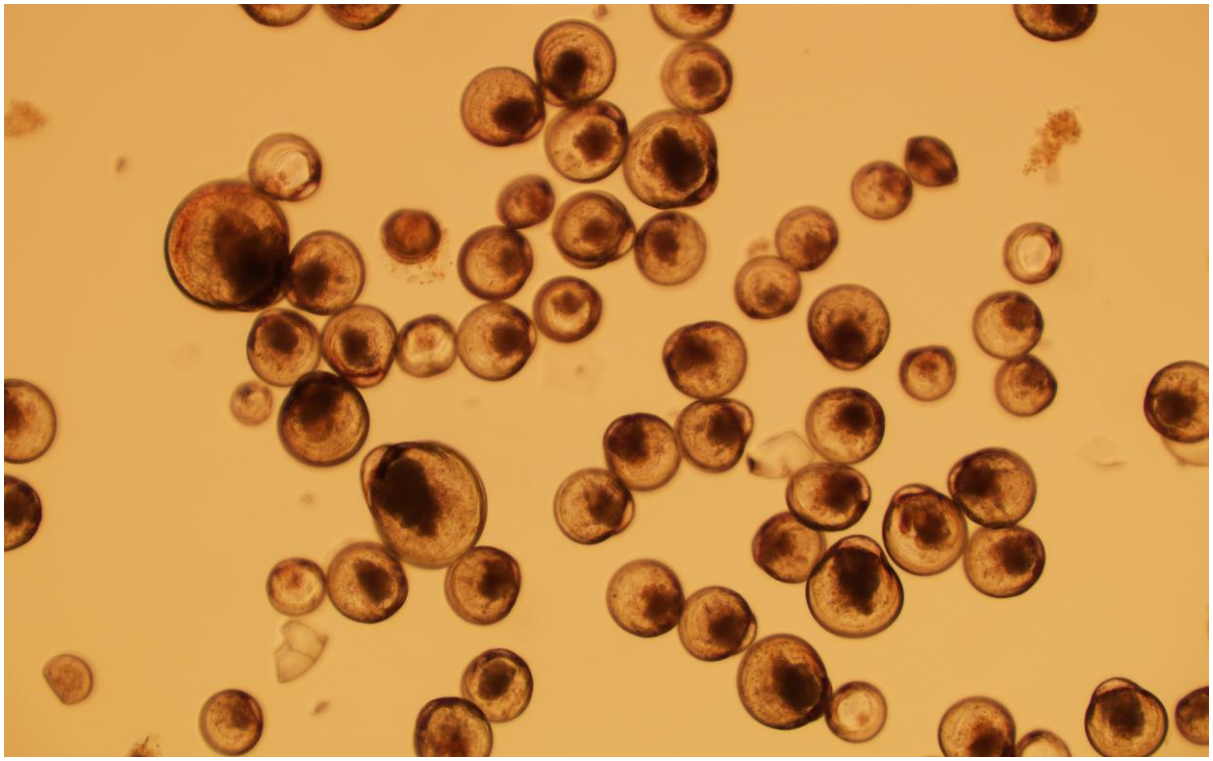
Uren Webster, T. M., Consuegra, S., & Garcia de Leaniz, C. (2021). Early life stress causes persistent impacts on the microbiome of Atlantic salmon. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, 40. https://doi.org/10.1016/j.cbd.2021.100888

Utting, P.F. (1997). Millican,Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability, *Aquaculture Volume,* 155 (1–4), 45-54. https://doi.org/10.1016/S0044-8486(97)00108-7.

Vignier, J., Laroche, O., Rolton, A., Wadsworth, P., Kumanan, K., Trochel, B., Pochon, X., & King, N. (2021). Dietary Exposure of Pacific Oyster (*Crassostrea gigas*) Larvae to Compromised Microalgae Results in Impaired Fitness and Microbiome Shift. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.706214

Vogeler, S., Bean, T. P., Lyons, B. P., & Galloway, T. S. (2016). Dynamics of nuclear receptor gene expression during Pacific oyster development. *BMC Developmental Biology*, 16(1), 1–13. https://doi.org/10.1186/s12861-016-0129-

Chapter four: Manipulation of the *Crassostrea gigas* larval microbiome via temperature and diet.



Abstract:

The cultivation of *Crassostrea gigas* (the Pacific oyster), produces an important source of global protein. However, the overuse and misuse of antibiotics within oyster hatcheries has created the need for alternative methods of maintaining disease-free larvae and stable healthy microbiomes. This chapter investigates the impact of temperature and algal feed on larval microbiomes and their potential for manipulation of the microbiome. Pacific oyster larvae were reared until 15 days old under temperatures of 20°C vs 25°C and diets of *Tisochrysis lutea* vs *Tisochrysis lutea* in combination with *Tetraselmis suecica*. Samples of algae, larvae and larval culture water were collected and total culturable bacterial load, bacterial diversity and larval growth rate measurements were recorded between the different treatment groups. Larvae grown at the higher temperature of 25°C showed an increased growth rate. However, insufficient amplification of bacterial DNA meant that specific bacterial taxa between treatment groups could not be analysed. Larvae grown under a diet of *Tetraselmis suecica* and *Tisochrysis lutea* also showed improved growth rates compared to those grown with just *Tisochrysis lutea.* The microbiomes of larvae grown under these two dietary treatments were found to be distinct from one another, with microbial differences being driven by the abundances of certain taxa such as *Actinobacteria, Bdellovibrionia* and *Bacilli* and not species richness. The majority of taxa found within larval samples were also detected in the algal feed, suggesting that the bacteria present in algae is a likely to be a key source of the larval microbiome.

Introduction:

The importance of oyster aquaculture:

In order to provide sufficient protein for a rapidly growing global population, aquaculture production has increased dramatically, with output now surpassing that of capture fisheries (Botta et al, 2020). Indeed, the FAO estimates seafood production (including shellfish) from both aquaculture and capture fisheries to contribute 17% of the world's total animal protein (Boyd, McNevin and Davis, 2022; FAO, 2020). The farming of mollusc species makes up the second largest category of farmed seafood in both quantity and value (Botta et al, 2020; Pernet et al, 2016). More specifically, *Crassostrea gigas* (the Pacific oyster) represents 98% of oyster production (Petton et al, 2021) with worldwide production reaching 643,500 tons in 2018 (Martínez-García et al, 2022). This species grows rapidly, has broad marketability and, tolerance to a wide range of environmental conditions. This has resulted in it being farmed all over the world, with top producers including China, France, South Korea and Japan (Botta et al, 2020; Salgueiro et al, 2021). Oyster farming therefore not only contributes to global food security, but provides these countries and many others with a direct source of employment and economic growth.

Oyster aquaculture is reliant upon the production of seed stock:

A common theme in oyster aquaculture across the world, is the reliance of the industry upon hatchery-produced seed stock (Allen et al, 1993; Hershberger et al, 1984). Seed stock is a term used to refer to young oysters (also often referred to as spat), which have recently undergone the settlement phase of the lifecycle. During this settlement process, oyster larvae progress from free-swimming within the water column, to small sessile oysters with a hard outer shell (Vogeler et al, 2016). In many countries, quantities of natural spatfall are unable to fulfil market demands (Humphreys et al, 2014) and so seed stock are commonly produced in hatcheries (Yeh et al, 2020). It is the production of this seed stock that is then competitively sold to commercial oyster farms where they can mature before reaching market size. Crucially, oyster aquaculture is dependent upon seedstock production being both of a high quality and in consistent, reliable supply (Martínez-García et al, 2021).

Mortality events are the most significant challenge to oyster seed stock production:

Oyster hatcheries routinely suffer from large-scale disease mortalities caused by bacteria (Richards et al, 2015; Barbosa Solomieu et al, 2015; Petton et al, 2021), viruses (Friedman et al, 2005; Humphrey et al, 2014; King et al, 2012; Barbose Solomieu et al, 2015), parasites (Enríquez-Espinoza et al, 2010) or fungi (Borzykh and Zvereva, 2012; Elston et al, 2012). These mortality events are often complex, multi-factorial events, and can cause losses of up to 100% of seedstock. By limiting the production of seedstock that the industry is so reliant upon, these mass mortality disease events are significantly limiting oyster production and the growth of aquaculture. Understanding such disease events and larval health in general is therefore crucial to improving oyster cultivation.

Larval health and the microbiome:

An important aspect of oyster larval health is their microbiome. A microbiome is the collection of microorganisms including bacteria, viruses and fungi that reside within an environment and is understood to play crucial roles in host health, including protection against disease (Infante-Villamil et al, 2021; King et al, 2012; King et al, 2019; Lokmer and Wegner, 2015). More specifically in oysters, viable bacteria within the haemolymph have been shown to influence the disease status of animals by either inducing an immune response or competitive exclusion (Schmitt et al, 2012). The bacteria found within healthy *C. gigas* individuals is highly diverse and often beneficial to the host (Schmitt et al, 2012). However, should the microbiome become imbalanced, this can compromise the health of the oyster, often resulting in mortalities (Dugeny et al, 2022; Petton et al, 2021). This bacterial dysbiosis may occur through the introduction of pathogenic bacteria such as *Vibrio coralliilyticus* and *Vibrio tubiashii* (Richards et al, 2014). Alternatively, certain environmental conditions may encourage bacterial species which previously were a part of a healthy microbiota to become opportunistic pathogens (Lokmer and Wegner, 2015). The oyster larvae microbiome is therefore intrinsically linked with their environmental microbiomes and disease outcomes. Since most reported bacterial disease events occur during the larval stages (Garnier et al, 2007), microbiome studies predominantly focus on this stage of the life cycle.

Current methods of larval microbiome manipulation:

Although under strict regulation, oyster hatcheries are able to introduce antibiotics such as vancomycin, ampicillin and streptomycin into their larval cultures in order to maintain disease-free larvae (Kang et al, 2018). Whilst such a strategy is immediately effective in reducing the abundance of bacteria, overuse of antibiotics is known to lead to antibiotic-resistant pathogens (Sun et al, 2020). For example, decreased susceptibility to oxytetracycline has been reported in microbes from all bivalve species (Salgueiro et al, 2021). Similarly, Kang et al, 2017, reports a positive correlation between an increase in antimicrobial usage and isolation rate of antimicrobial- resistant *Vibrio parahaemolyticus*. Yeh et al, 2020 also points to growing consumer concern over the use of antibiotics in farming potentially damaging sales in shellfish. There are, however, alternative methods of microbiome manipulation in shellfish, for example through the means of probiotic treatments(Ringø 2020). Whilst these treatments are promising for improving growth performance and reducing the overall impact of disease, few probiotics are targeted specifically towards oyster cultivation. Indeed, Yeh et al, 2020 states that the transfer of probiotics used for other aquaculture species into marketable products for bivalves to be a slow and complex process. With major limitations to the use of antibiotics, the development of alternative ways to control to larval microbiome is a crucial element to sustainable shellfish aquaculture.

Microbiome manipulation via diet and temperature control:

Considering the limitations of antibiotic usage along with current underdeveloped probiotic strategies, we investigated methods of larval microbiome manipulation via diet and temperature control, both aspects of larval husbandry that any hatchery would be able to implement. As filter feeders, oyster larvae are closely linked to their external environments (King et al, 2019). This therefore gives high potential for their own microbiota to be influenced by the parameters and bacteria present in their surrounding seawater.

Oyster hatcheries commonly rear larvae between 25-28°C (Meng et al, 2021), with the rationale that this temperature results in an optimal growth rate. There is however a strong basis within the current literature that heat shock events are linked with proliferation of pathogenic *Vibrio* species and *C. gigas* larval and spat mortality events (Green et al, 2019,

Siboni et al, 2024). Green et al, 2019 used a simulated heatwave event with an upper temperature threshold of 25˚C, and associated a 77.% oyster mortality rate to be caused by bacterial proliferation induced by the temperature increase. We therefore hypothesised that although an increased growth rate is achieved, higher water temperatures may also encourage the growth of bacterial species which could be detrimental to larval health and result in an overall less stable larval microbiome. This may ultimately increase the likelihood of a bacterial infection and large-scale mortality event. Should rearing larvae at a lower temperature result in a more beneficial host-associated microbiota, this may also have a secondary benefit of reduced operating costs during these phases of the growth cycle. We aimed to test this hypothesis by rearing oyster larvae at two different temperatures (20°C vs 25°C), and reporting both 16S rRNA sequence and colony forming units per mL (CFU/ml) data between these two different conditions. Growth rate measurements of larvae reared under both temperatures were also collected to confirm 25˚C to be the favourable condition in terms of growth rate.

In order to feed larvae, algae are routinely grown by oyster hatcheries and are consistently introduced into larval cultures as early as 24 hours post-fertilisation. The microalgae *Tisochrysis galbana (*also known as *Tisochrysis lutea),* is one of the most commonly produced, as it is small enough for young oyster larvae to feed on. There is also, however, anecdotal evidence that the larger species of algae *Tetraselmis suecica* is beneficial to larval growth despite being too large to ingest. This was used as rationale to hypothesise that the addition of *Tetraselmis suecica* could optimise larval growth not as a food source, but by altering the water chemistry and/or water microbiome and in turn potentially the larval microbiota. To test this, we reared oyster larvae under either a control diet (*Tisochrysis galbana*) or an experimental diet (*Tisochrysis galbana* and *Tetraselmis suecica).* Growth measurement data was collected across these two groups as well as CFU/mL and 16S rRNA sequence data. This chapter thus explores the potential of relatively minor husbandry changes to elicit favourable changes to the larval microbiome. These favourable changes to the microbiome will be evaluated through analysis of 16S species richness and composition data.

Rationale of bacterial identification methods used:

As previously discussed, this chapter mainly utilises long-read with Oxford Nanopore technologies 16S rRNA sequencing methods. This approach enabled higher throughput processing of samples while providing a greater change of improved taxonomic resolution in comparison to short-read methods. In addition to sequencing, bacterial identification of individual colonies isolated from larval samples in the diet manipulation experiment was performed using Matrix-Assisted Laser Desorption/Ionisation–Time of Flight (MALDI-TOF) mass spectrometry. MALDI-TOF was used to complement, rather than replace, 16S rRNA sequencing, providing a complimentary method for confirming bacterial identities with rapid and accurate protein-based profiling.

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Hypothesis tested:

1. Rearing oyster larvae at a higher temperature of 25˚C rather than 20˚C will result in impaired larval growth due to the presence of detrimental bacterial species.
2. The larval microbiome can be manipulated via diet.

Materials and methods:

Oyster spawning:

All oyster spawning, larval maintenance and experimental work was conducted within the biosecure experimental facilities at the Centre for Environment, Fisheries and Aquaculture Science (Cefas) in Weymouth, UK. All seawater used throughout experiments was sourced locally from Newton's cove and processed via sand filter, before passing through two 1 µm cartridge filters and UV treatment. Seawater was maintained at a salinity of 34 ppt and temperature of 22°C unless stated otherwise.

Based on anecdotal evidence from shellfish practitioners, it is common practice for all seawater to be aged prior to use in larval cultures. This process aims to stabilise water quality parameters such as nutrient levels, metal ions and microbial compositions. To do this, filtered seawater was held in a 15 L conical tank with strong aeration for 24 hours prior to use.

Male and female broodstock oysters were supplied from Guernsey Sea Farms. These oysters had been pre-conditioned at Guernsey Sea Farms to ensure that they were 'ripe' and suitable for the purpose of broodstock. Oysters were held in separate tanks with 14°C filtered seawater for 12 hours prior to spawning (Figure 2a). Of these, 3 female and 2 male *Crassostrea gigas* (*C. gigas)* individuals were strip spawned via the scarification method at room temperature. This involves scarifying the gonad of the oyster with a sterile razor in order to expose the gametes which can then be collected by washing into seawater. Gamete quality was assessed considering the size and shape of eggs (Figure 1a), and the motility and presence of any abnormalities in the sperm. Gametes from each male and female were pooled in equal quantity (Table 1) and a final fertilisation of 3 million eggs was carried out at a 1:10 ratio with sperm. After 20 minutes, fertilised eggs were checked for the presence of polar bodies (Figure 1b) and allowed to incubate for 24 hours in 15 L glass conical tanks (Figure 2b – 2c) at a density of 50 eggs per mL with slight aeration at room temperature. After 24 hours, a subsample was taken for observation under a light microscope, and upon confirmation of D-shell larvae, tanks were restocked at a density of 10 larvae per mL. After further subsampling and counting of larvae, fertilisation success rate was estimated.

Table 1: Egg counts from each of the three *C. gigas* broodstock females used during spawning. Eggs were pooled equally to a final density of 3 million eggs.

Female number:	Count 1 (20 µL)	Count 2 (20 µL)	Count 3 (20 µL)	Average eggs/20 µL	Number of eggs/mL	Volume of eggs used in final pool (mLs)
1	142	164	140	148.6	7433	134
2	187	169	181	179	8950	112
3	323	331	294	316	15,800	63

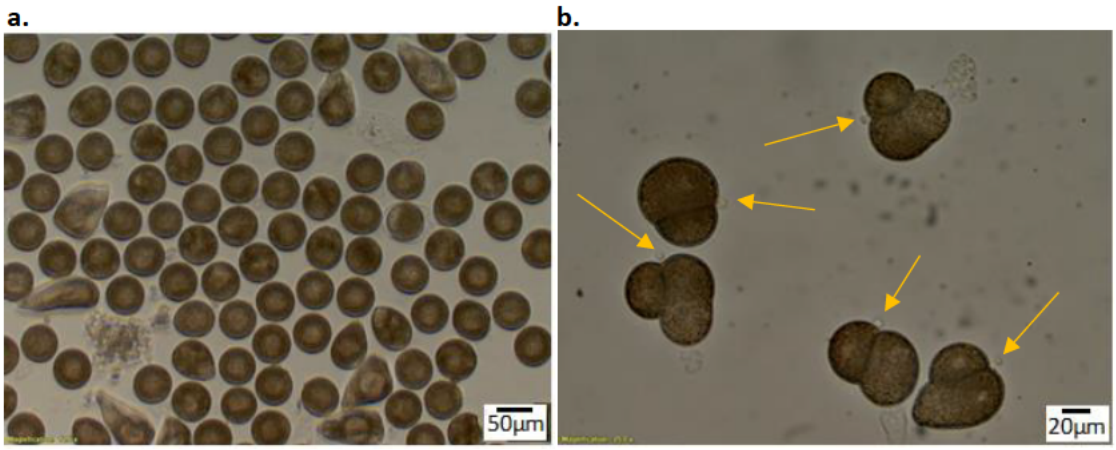


Figure 1: (a) Pooled sample of eggs from three *C. gigas* females used during spawning**. (b)** Early embryos from oyster spawning approximately 2 hours post fertilisation, with arrows indicating the position of polar bodies. Images taken on Olympus 1x83 microscope and camera.

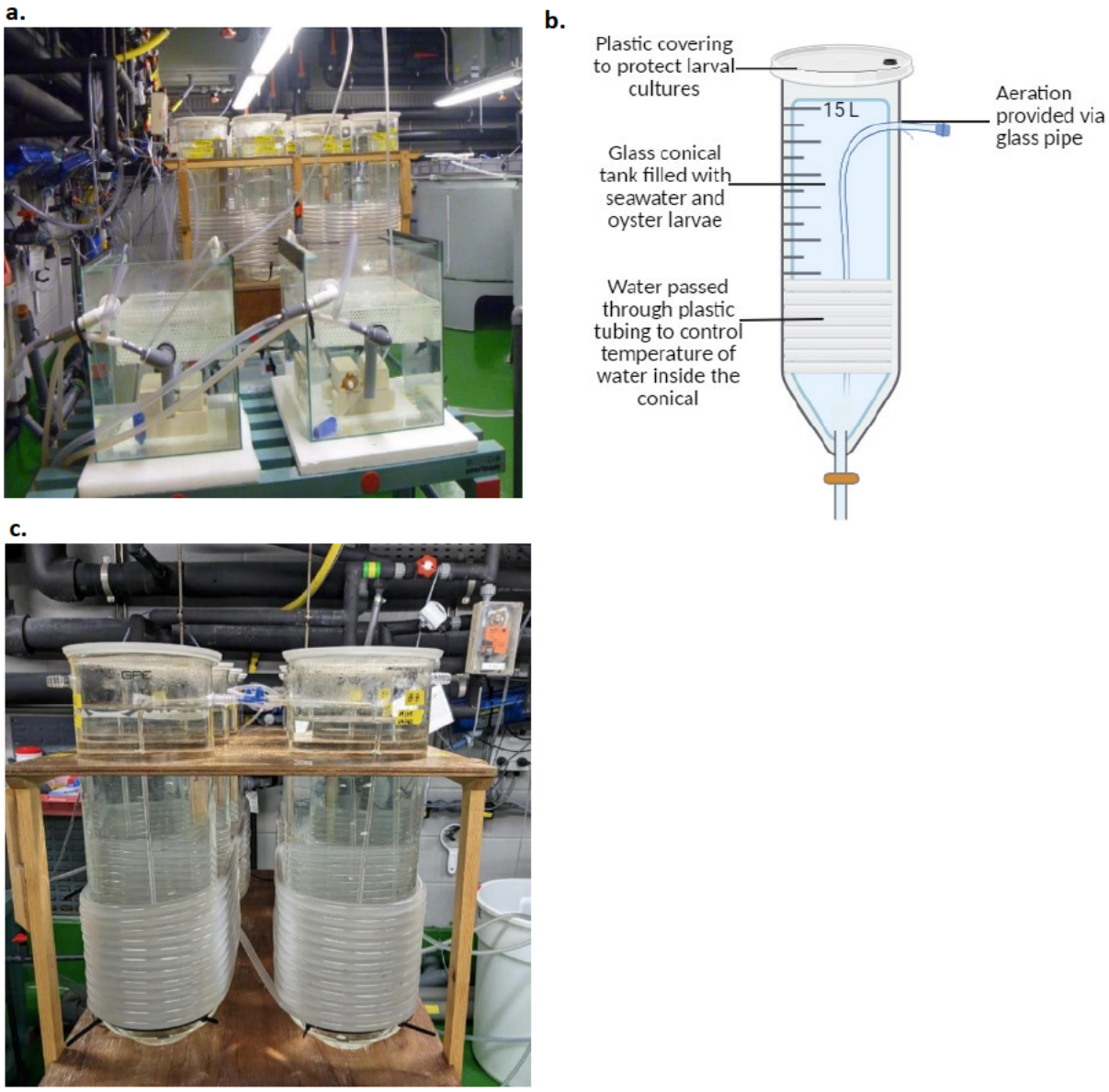


Figure 2: (a) Tanks used to hold male and female broodstock oysters separately in 14°C seawater prior to spawning. **(b)** Schematic diagram of a 15 L conical tank used in early larval rearing. **(c)** photograph of 15 L conical tanks used in early larval rearing. Created in https://BioRender.com

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Maintenance of larval cultures:

In order to maintain healthy oyster larvae, the seawater of larval cultures was changed 2-3 times a week. For experiments conducted in 15 L conical tanks, this was achieved by draining the tank onto a mesh sieve in order to collect oyster larvae (Figure 3) and discard old seawater. Oyster larvae could then be replaced into a clean conical tank filled with fresh seawater. All waste water was then ozone treated for appropriate disposal.

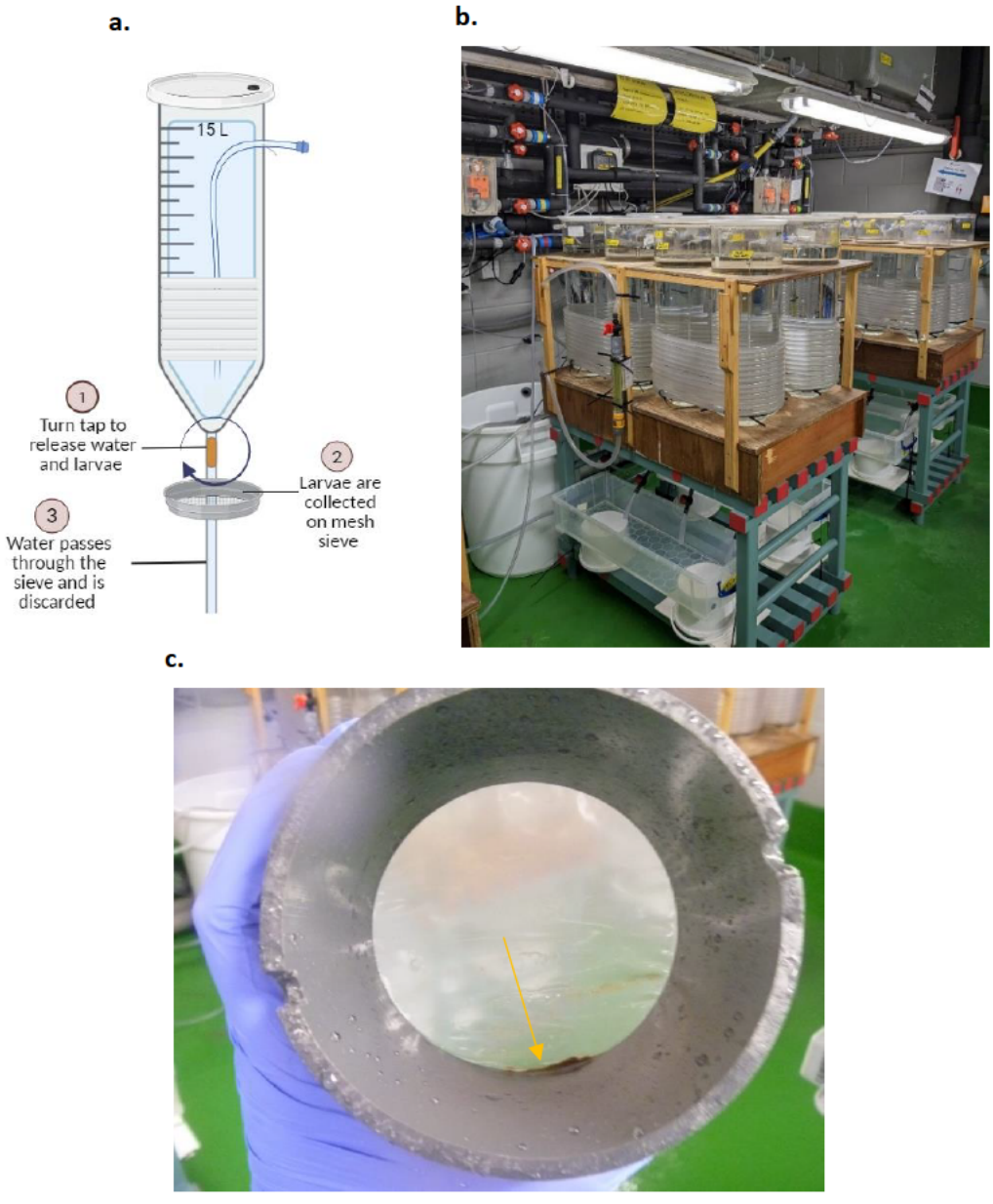


Figure 3: (a) Schematic diagram of a water change on a larval culture held in a 15 L conical tank. **(b)** Photograph of 15 L conical tanks. **(c)** Oyster larvae (indicated by arrow) condensed on a mesh sieve during a water change. Created in https://BioRender.com

Cultivation of algae:

In order to provide food and experimental treatment groups, starter cultures of *Tisochrysis lutea 927/19* and *Tetraselmis suecica 66/4* were purchased from the CCAP (culture collection of algae and protozoa). To produce a sufficient volume of each species, sub-culturing was carried out over a three-week period in 250 mL, 1 L and 4 L Erlenmeyer and round bottomed glass flasks. To subculture, algae was added in a 1:4 ratio with L1 growth media made with sterile seawater. Cultures were then grown under fluorescent grow lights with strong aeration delivered through a sterile glass pipette. The cell density of each culture was counted every 2-3 days with a haemocytometer. Once maximum cell density has been reached (after approximately 7-10 days), a new subculture was started in a larger volume (Figure 4).

Sample collection of algae:

Duplicate 1 mL samples of *Tisochrysis lutea* and *Tetraselmis suecica* were taken with a sterile glass pipette and stored in 1.5 mL Eppendorf's on the following dates (Table 2). One of the duplicate samples was immediately frozen at -20°C for future use in 16S sequencing. The remaining sample was then placed on ice for immediate use in calculation of CFU/mL.

Table 2: Sample collection dates of *Tisochrysis lutea* and *Tetraselmis suecica* where '☑' indicates the collection of one sample.

Date collected	*Tisochrysis lutea*	*Tetraselmis suecica*
16/06/2023	☑☑	☑☑
19/06/2023	☑☑	☑☑
22/06/2023	☑☑	☑☑
26/06/2023	☑☑	☑☑
29/06/2023	☑☑	☑☑

Calculation of CFU/mL from algal cultures:

Each algal sample was plated neat (100 µLs) onto marine agar and allowed to incubate at 20°C for 48 hours. Distinct colonies were then counted and recorded.

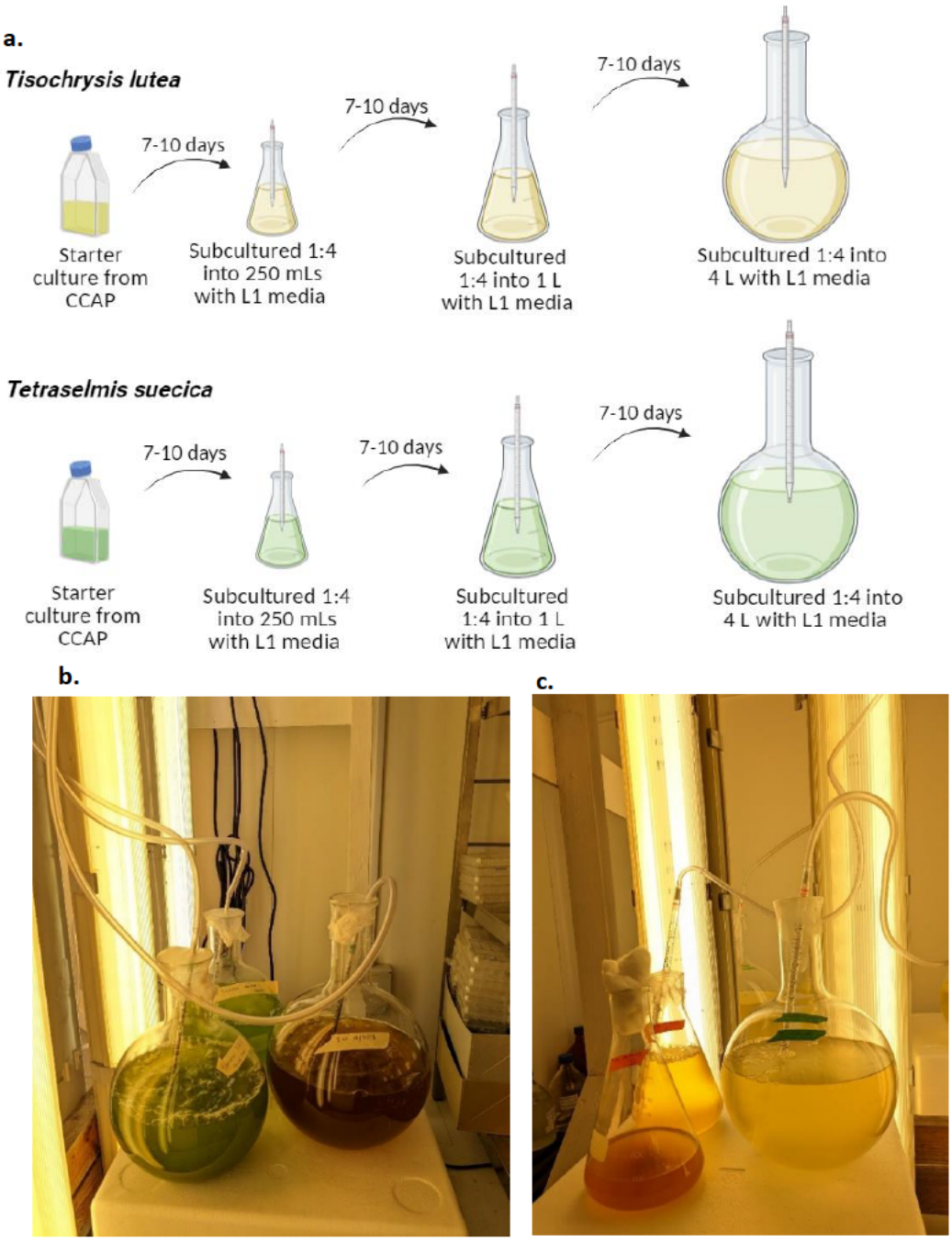


Figure 4: **(a)** Schematic showing the subculture process used to scale up algal production of Tisochrysis lutea and *Tetraselmis suecica* **(b)** Photographs showing the subculture process used to scale up algal production of *Tetraselmis suecica* and **(c)** *Tisochrysis galbana*. Created in https://BioRender.com

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Manipulation of the microbiome via temperature:

Experimental set up: Four-day old larvae were moved into 1 L glass beakers held in water baths maintained at either 20°C or 25°C (Figure 5). Each beaker was covered with a plastic lid, leaving a small gap for air exchange. This provides sufficient oxygenation to a smaller volume of 1L. Larvae were fed daily by adding *Tisochrysis galbana* in accordance with the feeding plan (Table 3). Water changes were carried out bi-weekly over the two-week course of the experiment. To do this, larvae were condensed on a 40 µm sieve and replaced into a clean beaker filled with 24-hour aged seawater, ensuring that separate equipment was used between treatment groups. S

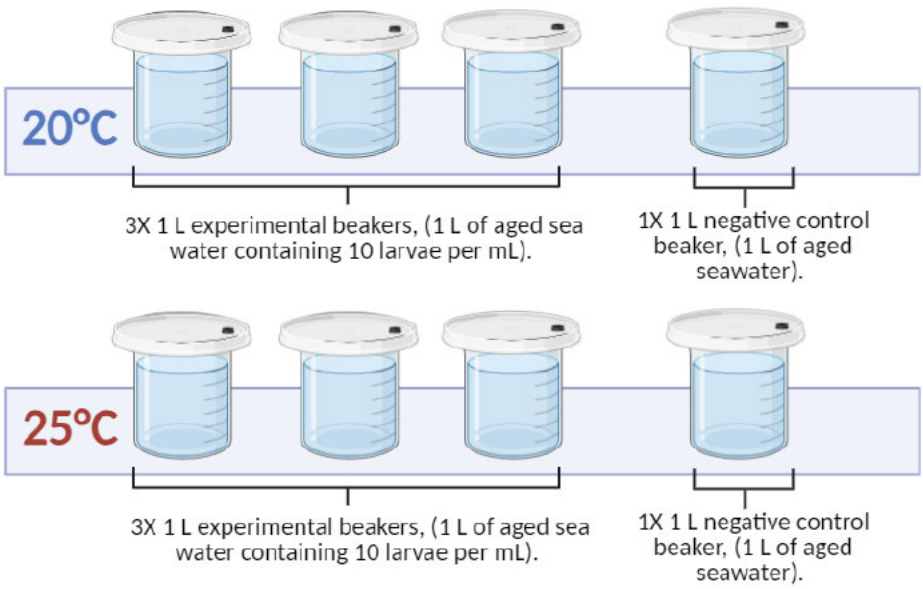
Table 3: Quantities of *Tisochrysis galbana* for each feeding event throughout the temperature manipulation experiment. Larvae were reared in 1 L glass beakers.

Age of larvae (days)	Algal cells (per µL of tank volume)	Overall number of algal cells per beaker
4-7	8	8,000,000
7-15	24	24,000,000

Sample collection:

After each water change, larvae were gently resuspended with a plastic plunger to ensure an even distribution within all of the beakers. Duplicate 1 mL samples of larvae (and their surrounding water) were then collected with a micropipette and stored in 1.5 mL Eppendorf tubes (Table 4). One replicate of each sample was immediately stored at -20°C for later 16S sequencing. The remaining replicate sample was placed on ice for immediate use in calculation of CFU/mL.

a.



b.

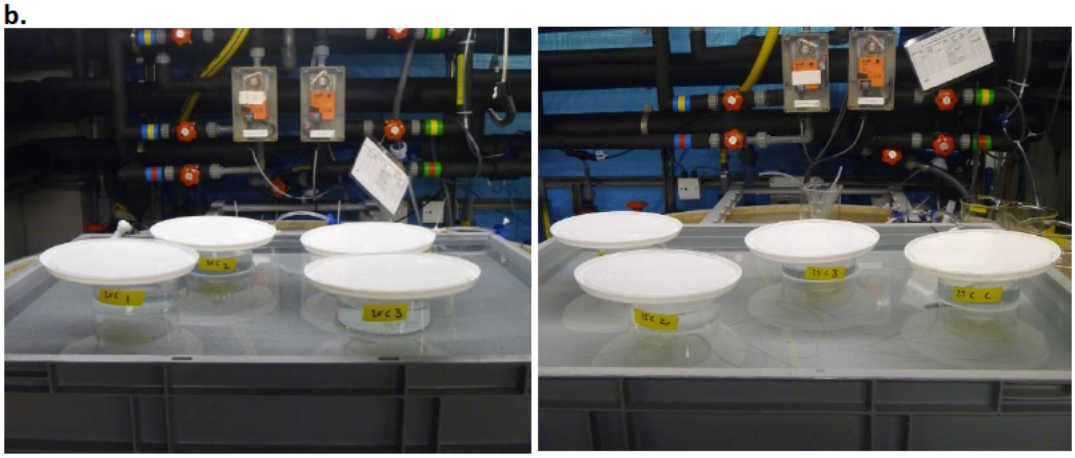


Figure 5: **(a)** Schematic of the experimental set up used for temperature manipulation of larvae. **(b)** Photographs of the experimental set up used for temperature manipulation of larvae showing 20˚C (left) and 25˚C (right). Created in https://BioRender.com

Table 4: Showing sample collection dates for larvae + surrounding water across treatment groups and replicates during temperature manipulation experiments, with '☑' representing the collection of one sample. A control beaker was included, consisting of a 1 L beaker filled with aged seawater (containing no larvae).

			20°C				25°C			
Date collected	Day of experiment	Age of larvae (days)	Rep 1	Rep 2	Rep 3	Control (water only)	Rep 1	Rep 2	Rep 3	Control (water only)
19/06/2023	1	4	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑
20/06/2023	2	5	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑
23/06/2023	5	8	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑
27/06/2023	9	12	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑
30/06/2023	12	15	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑	☑☑

Calculation of CFU/mL:

Each sample was diluted 1:100 with sterile saline and 100 µLs was plated onto marine agar. Plates were incubated at 20°C and distinct CFUs were counted after 48 hours. In this experiment, larval samples were not homogenised prior to plating due to the very low number of larvae present after centrifugation. This limited biomass created difficulties in concentrating the larvae sufficiently for homogenisation without risking the loss of material.

Growth measurements:

At the end of the two-week experiment on 30/06/2023, a 1 mL sample of water and larvae was taken from each beaker using a micropipette. Larvae were then transferred into a 6 well plate for observation under an Olympus 1X83 microscope and camera. Anterior to posterior measurements of 40 larvae per beaker were taken and automatically recorded using digital micrometre measurement tools on the microscope. These data were then used to calculate average growth measurements for larvae across both of the treatment groups (20°C vs 25°C).

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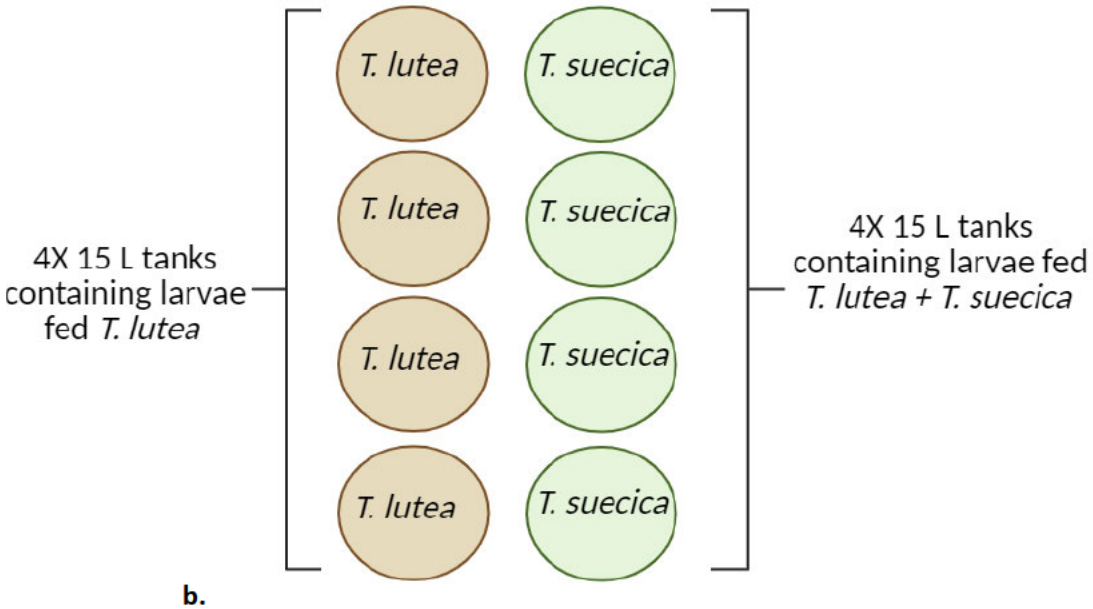
Manipulation of the microbiome via diet:

Experimental set up: One-day old larvae were distributed across eight 15 L glass conical tanks at a density of 10 larvae per ml and held with slight aeration delivered through a glass pipe (Figure 3a). Aeration via this method is necessary to deliver sufficient oxygenation to the larger volume of 15L. A total of four replicate tanks were used across two different treatment groups, with larvae being fed either a control diet of *Tisochrysis lutea* or an experimental diet of *Tisochrysis lutea* and *Tetraselmis suecica* in accordance with the following feeding plan (Table 5). Before adding to the larval cultures, algae were passed through a 20 µm mesh sieve in order to remove any debris, ensuring that different sieves were used between different treatment groups.

Table 5: Quantities of *Tisochrysis galbana* and *Tetraselmis suecica* given to larvae throughout the diet manipulation experiment. Larvae were reared in 15 L tanks.

Age of larvae (days)	Volume of *Tisochrysis lutea* fed daily (algal cells per µL of tank volume)	Volume of *Tetraselmis suecica* fed daily (algal cells per µL of tank volume)
1-7	8	0.3
8-14	24	8

a.



b.



Figure 6: (a) Schematic diagram showing 15 L conical tank replicates incorporating two different diet-based treatment groups (*Tisochrysis lutea) and (Tisochrysis lutea with Tetraselmis suecica.)* **(b)** Photograph of 15 L conical tank replicates incorporating two different diet-based treatment groups (*Tisochrysis lutea)* and *(Tisochrysis lutea with Tetraselmis suecica).*

Sample collection:

Regular water changes of larval cultures also provided opportunity for sample collection. During the draining of each tank, duplicate water samples (containing no larvae) were collected. Before replacing larvae back into conical tanks of fresh seawater, they were first transferred into a 500 mL measuring cylinder. Larvae were gently mixed with a plastic plunger to ensure even distribution before sampling. Duplicate samples of oyster larvae were then collected into 1.5 mL Eppendorf's using a micropipette (Table 6). One sample from each replicate was immediately frozen at -20°C for future use in 16S sequencing, whilst the remaining replicate was placed on ice for use in CFU/mL calculations the same day.

Calculation of CFU/mL:

All larval samples were spun at 1000 rpm for 2 minutes in order to condense the larvae and discard their surrounding water before further processing. Larvae were then homogenised using a sterile plastic pestle in the bottom of the Eppendorf. This was possible since this experiment used large culture volumes (15L), collecting more larvae per sample. The use of homogenisation was intended to capture microbiota from within the larvae itself. Serial dilutions with sterile saline were then made and 100 µLs were plated onto marine agar. Serial dilutions of larval culture water and 24-hour aged water were also made and plated onto marine agar. All plates were then incubated at 20˚C and distinct CFUs counted after 48 hours.

MALDI-TOF Mass Spectrometry:

Distinct bacterial colonies grown from 13-day old larvae were selected for bacterial identification using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). In total, eight CFUs were tested (in duplicate), with four grown from larvae fed *Tisochrysis lutea*, and four grown from larvae fed *Tisochrysis lutea* and *Tetraselmis suecica*. To prepare for this analysis, a sample of bacteria was picked from each colony using a sterile wooden toothpick and spread onto a designated area of a MALDI-TOF plate before allowing to dry at room temperature for two minutes. A Cyano-4-hydroxycinnamic acid (HCCA) matrix solution was then prepared and added to the dried bacterial samples in accordance with the manufacturer's instructions. The MALIDI-TOF plate

was then loaded into the MALDI-TOF machine for identification of the bacterial colonies selected.

Growth rate measurements:

At the final time point, larvae from each conical tank were sampled and transferred into a 6 well plate for observation under an Olympus 1X83 microscope and camera. Using measurement tools on the microscope, anterior to posterior measurements of 40 larvae per beaker were recorded. These measurements were then used to calculate average growth measurements for larvae across both of the treatment groups.

Table 6: Sample collection throughout the diet manipulation experiment, incorporating samples of larval culture water (☑), larvae (☑) and 24-hour aged water (☑) taken on 5 different dates and across two different treatment groups (*Tisochrysis lutea vs Tisochrysis lutea* + *Tetraselmis suecica).*

			Tisochrysis lutea				*Tisochrysis lutea + Tetraselmis suecica*				
Date collected	Age of larvae (days)	Day of experiment	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	24-hour aged seawater
16/06/2023	1	1	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑
19/06/2023	4	4	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑
22/06/2023	7	7	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑
26/06/2023	11	11	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑
29/06/2023	14	14	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑☑☑	☑☑

Molecular biology:

Library preparation and sequencing was carried out as previously described in Chapter three. The pooled library was loaded onto an ONT MinION R10 flow cells for 48 hours, obtaining a total of 221 GB raw data.

Data handling and analysis:

Basecalling was performed using Guppy v.6.5.7 (Oxford Nanopore Technologies). Quality control summaries were generated using with pycoQC v 2.5.0.3 (Leger and Leonardi, 2020), reporting 3,780,147 reads with a mean read length of 1,552 and median PHRED quality score of 10.58. Primer trimming was then preformed using Cutadapt v.4.8 (Martin, 2011), with a maximum error rate of 0.15 and minimum overlap of 20 base pairs (bp). Reads shorter than 1,300 bp and longer than 1,600 bp were also discarded. Porechop v0.2.4 (Wick et al, 2017) was used to trim reads of chimeras and adapter sequences. Microbial taxonomy was identified using Kraken2 v2.1.1 (Wood and Salzberg, 2014), where reads were aligned against the SILVA138 database (Wood, Lu and Langmead, 2019).

Downstream data analysis was carried out in R studio v4.2.0 (RStudio Team, 2022) using the Phyloseq (McMurdie and Holmes, 2013) ggplot2 (Wickham, 2014), readxl (Wickham and Bryan, 2019), dplyr (Wickham et al, 2012) and tibble (Müller and Wickham, 2023) packages. After excluding positive and negative controls, 34 samples remained in the final analysis. Measurements of beta diversity were calculated using Bray-Curtis dissimilarity metrics visualised through non-metric multidimensional scaling (NMDS) and statistically evaluated using a PERMANOVA test using the Vegan package (Oksanen, 2012). Measurements of alpha diversity (both observed and Chao 1) were also calculated and tested for statistical significance with a Kruskal Wallis test. Weighted phylogenetic tress (Figures 3 and 6), were created using the Pavian web application tool (Breitwieser and Salzberg, 2020). GraphPad Prism (v10.3.1) was used visualise growth rate data and test for statistical significance with an unpaired t-test.

Results:

Manipulation of the microbiome via temperature:

At the final time point, when larvae were 15 days old, those grown at 25˚C were significantly larger than those grown at 20˚C (p = 0.0082), (Figure 7). Larvae grown at 20˚C had an average anterior to posterior measurement of 155.6 µm, whereas those grown at 25˚C had an average measurement of 192.4 µm. Photographs of example larvae measured across these two experimental groups are shown in Figure 8.

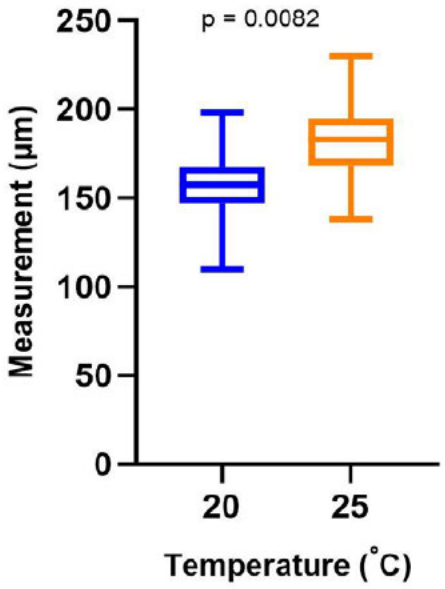


Figure 7: Growth measurements of 15-day old larvae reared at 20˚C versus 25˚C. Three experimental replicates are accounted for within each treatment group. The results of an unpaired t test between the two groups report a p value of 0.0082 (t= 4.874, df=4).

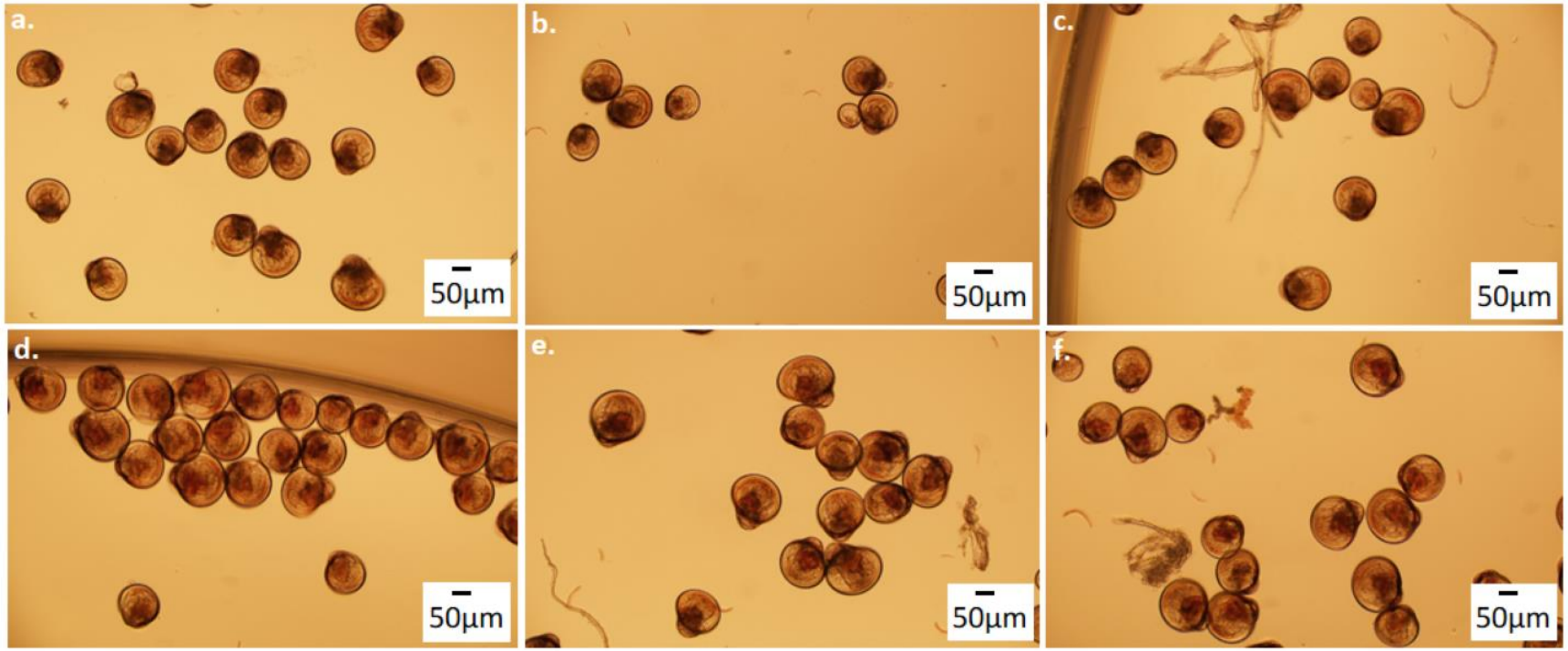


Figure 8: Larvae 15 days post spawning from cultures grown at 20°C **(a, b, c)**, versus 25°C **(d, e, f)**.

Abundance levels of culturable bacteria were calculated via CFU/mL from samples of larvae (not separated from their surrounding water) grown at 20°C vs 25°C. At the final time point, larvae reared at 25°C gave an average of 1.088 x 10^3 CFU/mL (calculated between three replicates), whereas those reared at 20°C resulted in slightly fewer at 9.29 X 10^2 CFU/mL (calculated between three replicates). Single samples taken from negative control beakers (seawater containing no larvae), gave slightly higher CFU/mL values with 1.084 X 10^3 CFU/mL recorded from water maintained at 20°C vs 1.220 X 10^3 CFU/mL from water maintained at 25°C (Figure 8). Photographs of CFUs on marine agar obtained from samples of 5-day old larvae are also shown (Figure 9), with negative controls of 20°C and 25°C experimental groups highlighted in red.

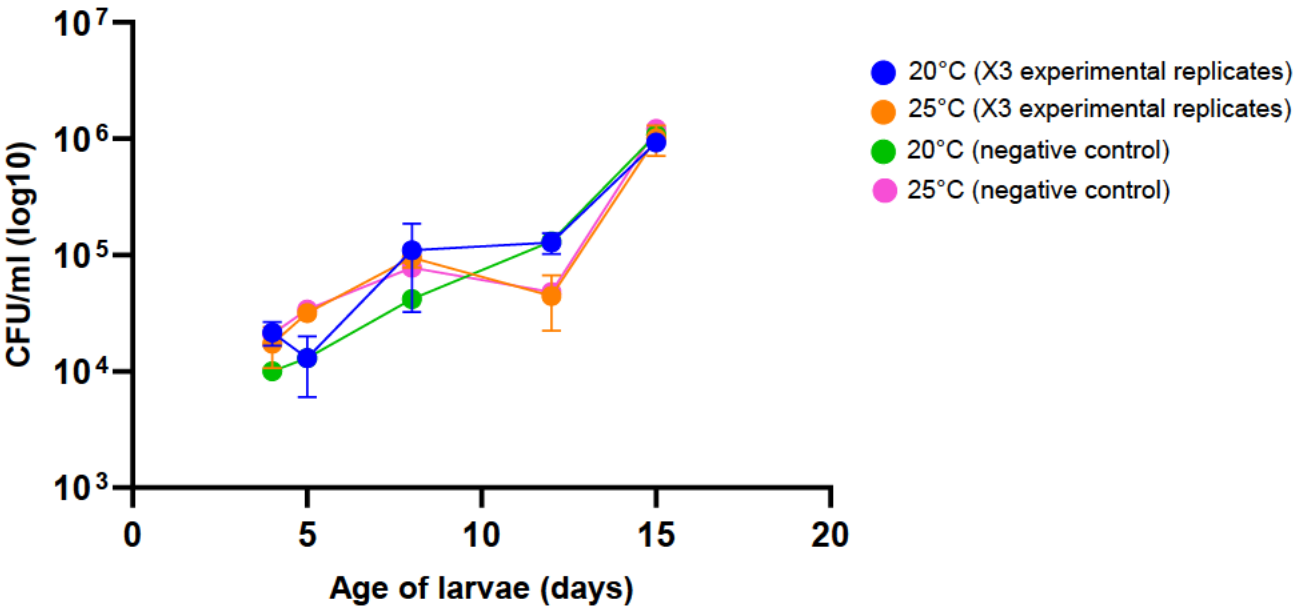


Figure 9: CFU counts from cultures of larvae at days 4, 5, 8, 12 and 15 days old. Larvae grown at 20˚C are represented in blue, and those grown at 25˚C in orange. CFU counts from negative control beakers (seawater containing no larvae) are shown in green (20°C treatment group) and pink (25°C treatment group).

a. **b.**

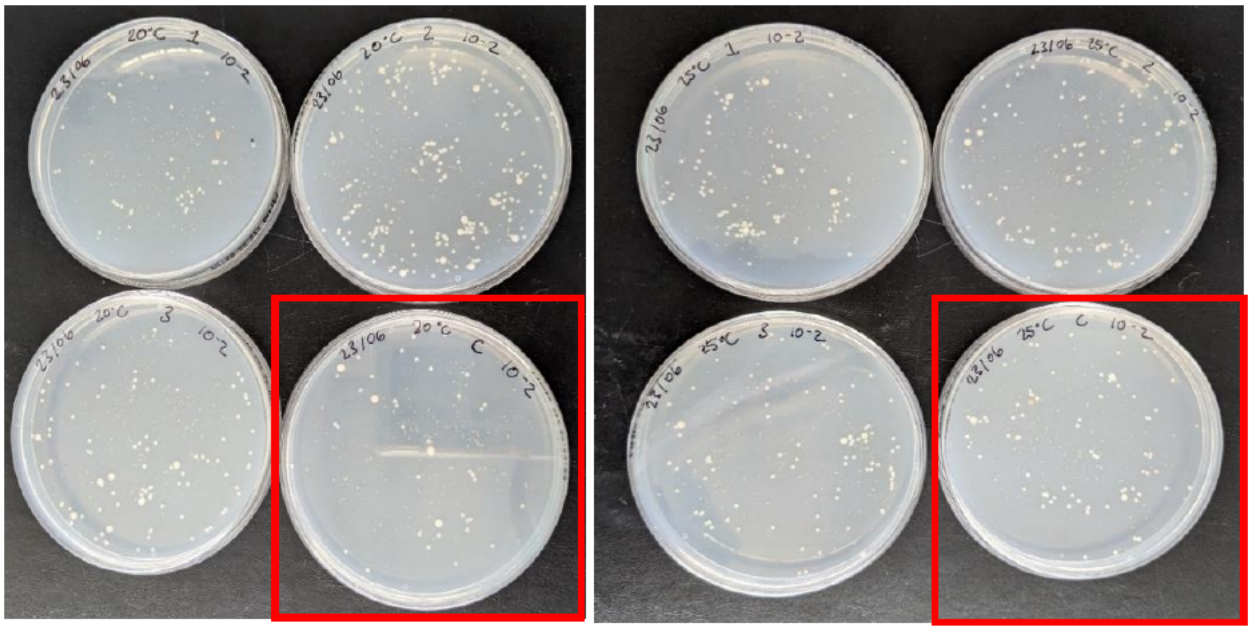


Figure 10: CFUs on marine agar from samples of larvae (and their surrounding water) reared at **(a)** 20°C vs **(b)** 25°C and their surrounding water. CFU counts from control beakers (containing no larvae) are highlighted in red.

Manipulation of the microbiome via temperature (16S sequence data):

Samples were taken throughout the temperature experiments with the intent to analyse *via* 16S sequencing. Analysis of amplified bacterial DNA by gel electrophoresis and selected clean-up and quantification demonstrated that all samples collected throughout the temperature manipulation experiment yielded insufficient amplification of 16S amplicon for nanopore sequencing. As such, this was not taken further.

* * *

Results: Manipulation of the microbiome via diet:

Growth and CFU measurements from algal cultures:

The cell densities of *Tisochrysis lutea* and *Tetraselmis suecica* cultures were counted every one to three days (Figure 11a). In order to ensure that algal cultures remained healthy and with sufficient growth media, *Tetraselmis suecica* cultures were sub-cultured on day 7 and *Tisochrysis lutea* on day 10. Cell densities recorded from *Tisochrysis lutea* cultures remained consistently higher than those of *Tetraselmis suecica* with final cell-densities on day 16 being 1.08×10^7 and 1.95×10^6 cells per mL retrospectively.

CFU counts from cultures of both algal species were also recorded over time (Figure 11b). Both *Tisochrysis lutea* and *Tetraselmis suecica* remained free of any culturable bacteria up to day 5, with no CFUs being counted up to this day. Between days 5 and 15, the culturable bacterial load in both species of algae increased, with *Tisochrysis lutea* cultures containing 4.30×10^2 CFU/mL and *Tetraselmis suecica* cultures containing 9.0×10^2 CFU/mL at the final time point of 15 days (Figure 11b).

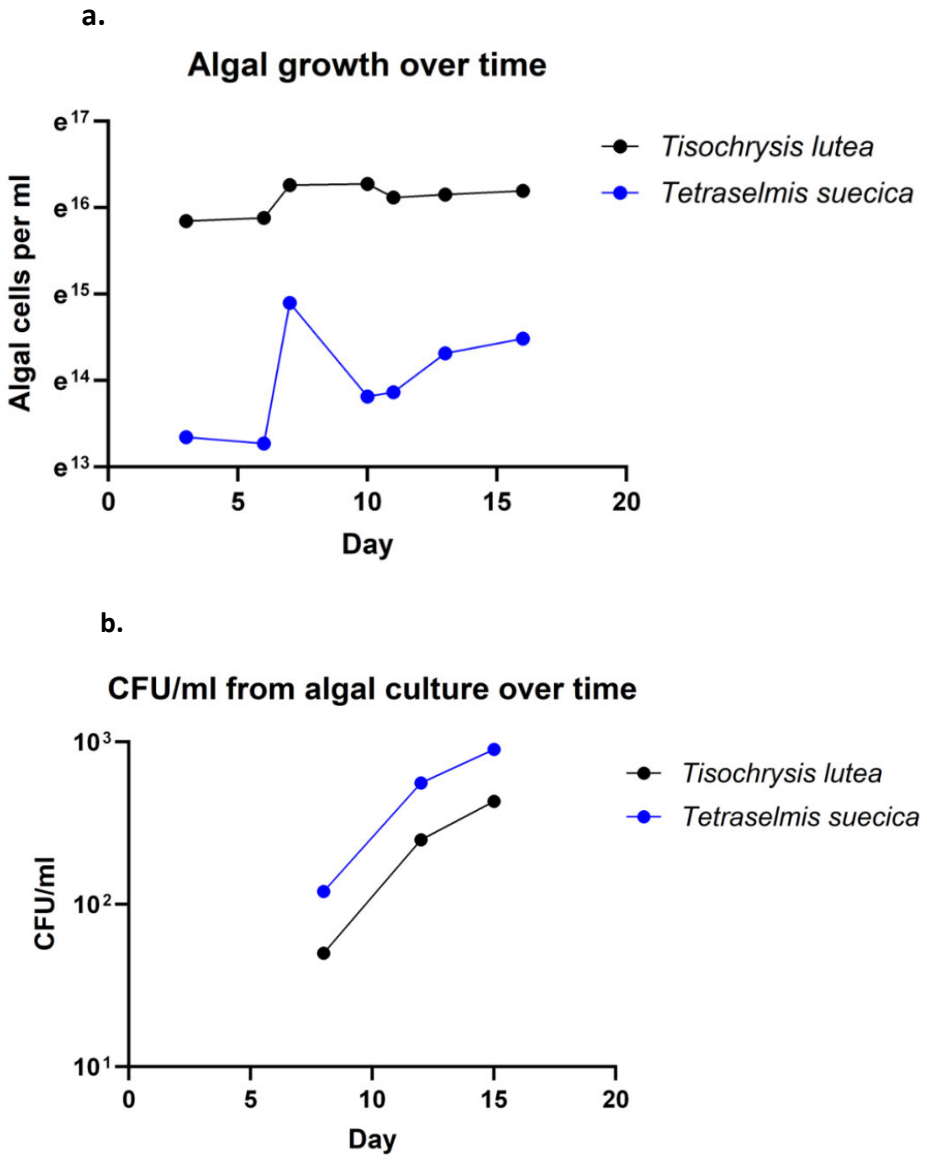


Figure 11: (a) Growth of *Tisochrysis lutea* and *Tetraselmis suecica* cultures over time. *Tetraselmis suecica* cultures were sub-cultured on day 7 and *Tisochrysis lutea* on day 10. (**b)** CFU/ml obtained from cultures of *Tisochrysis lutea* and *Tetraselmis suecica* over time.

Growth and CFU measurements from larval cultures:

Larvae fed only *Tisochrysis lutea* had an average anterior to posterior measurement of 133 µm after 14 days, whereas those which were also fed *Tetraselmis suecica* had an average measurement of 148.21 µm (p= <0.0001), (Figure 12). Examples of larvae measured across these two treatment groups can be seen in Figure 13.

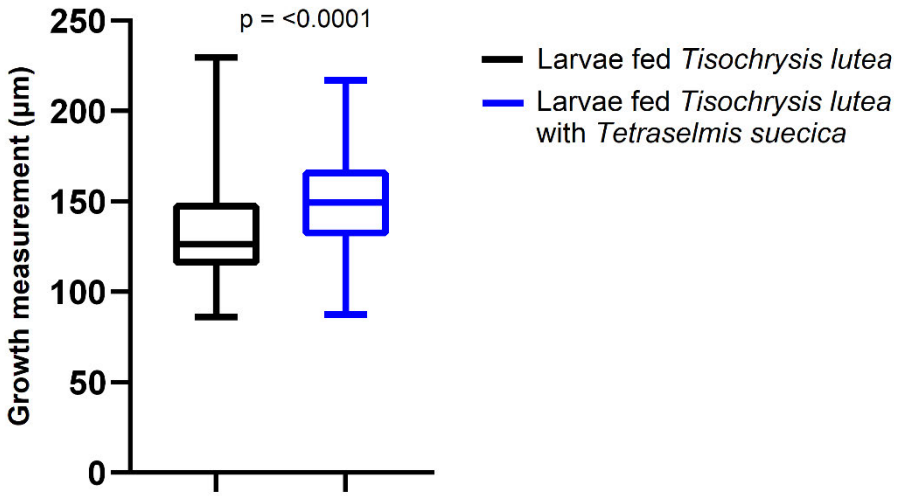


Figure 12: Anterior to posterior measurements from 14-day old larvae cultivated with *Tisochrysis lutea* (black) and *Tisochrysis lutea* with *Tetraselmis suecica* (blue). A total of 240 larvae were measured, including four replicates of 60 larvae from each treatment group. An unpaired t test between the two groups reports a p value of <0.0001 (t=5, df = 238).

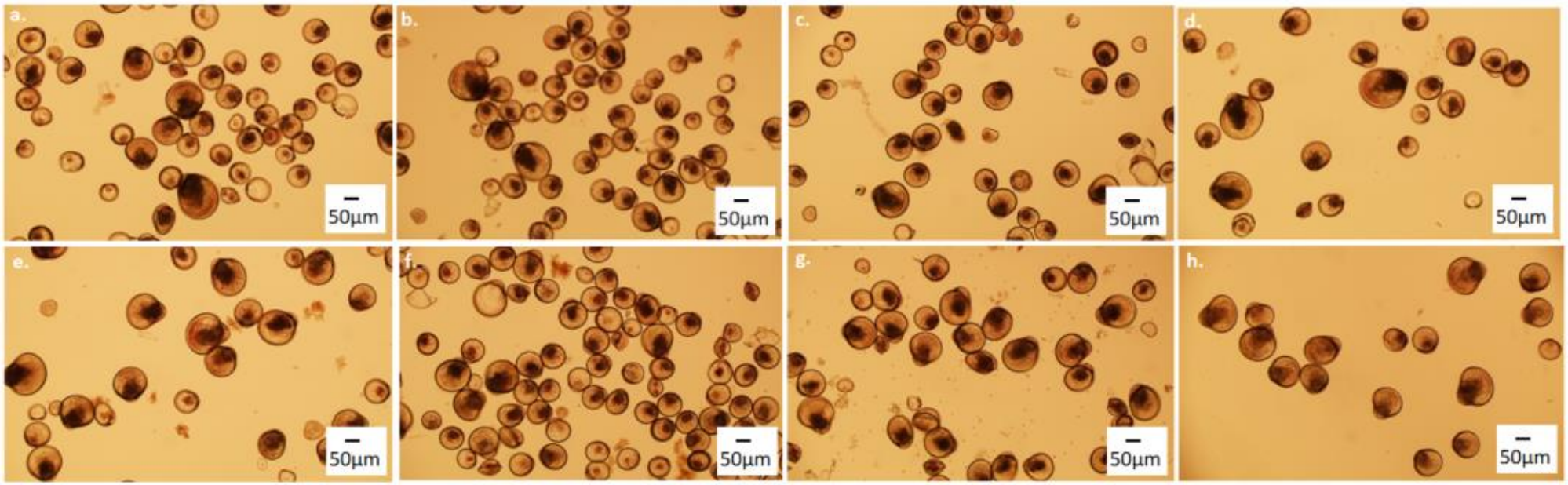


Figure 13: 14-day old larvae after being cultured with *Tisochrysis lutea* **(a, b, c, d)** versus *Tisochrysis lutea* and *Tetraselmis suecica* **(e, f, g, h)**.

The number of CFU/mL obtained from larvae at 4 and 14 days old under the two dietary treatment groups is shown below (Figure 14a). Little difference is seen between the CFU/mL from larvae grown under different dietary exposures, however CFU/ml did increase between larvae at age 4 to 14 days old. CFU counts from larval culture water (containing no larvae) also showed little variation between dietary treatment groups (Figure 14b). All bacterial colonies were observed to have of small, flat, white morphologies. Of the eight colonies chosen for MALDI-TOF analysis, bacteria were identified to be either *Vibrio fortis* or *Vibrio pelagius* (Table 7).

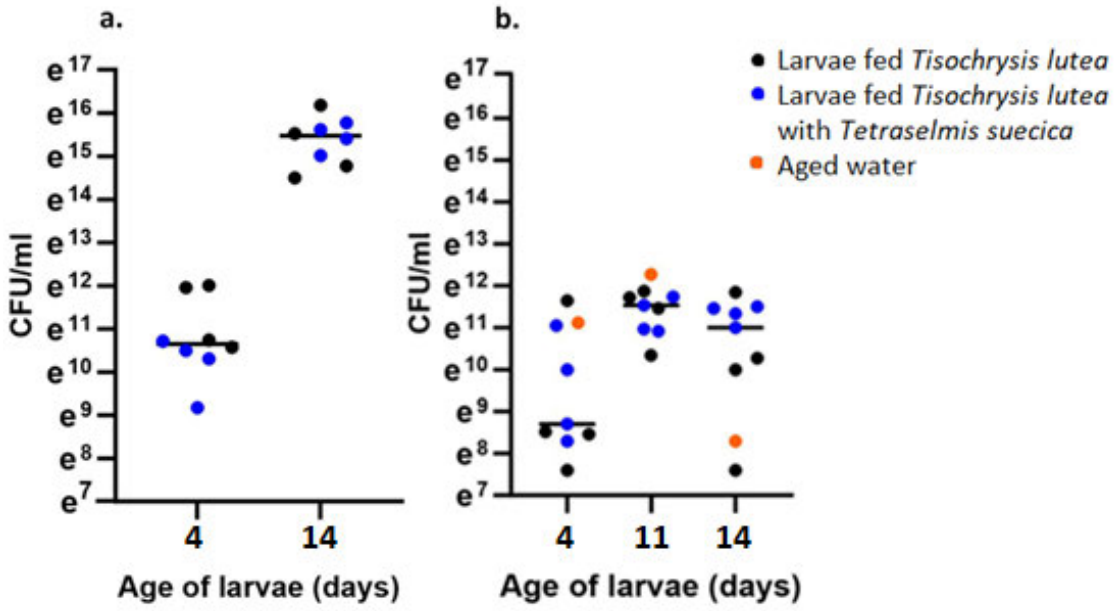


Figure 14: CFU/mL recorded throughout days 4 to 14 of oyster cultivation. **(a)** CFU/mL obtained from oyster larvae given the control diet (*Tisochrysis lutea*) shown in black versus the experimental diet (*Tetraselmis suecica*) shown in blue. **(b)** CFU/mL from larval cultivation water across treatment groups alongside 24-hour aged seawater.

Table 7: MALDI-TOF report results of bacterial identification from CFUs grown from 14-day old larvae cultivated under two different dietary treatment groups (A = *Tisochrysis lutea* versus B= *Tisochrysis lutea* and *Tetraselmis suecica*). Eight bacterial colonies were tested in total, each in duplicate.

Replicate number	Treatment group	Organism (best match)	log(score) (Conf.)	Organism (second-best match)	Log(score) (Conf.)
Larvae (replicate 1)	A	*Vibrio pelagius*	2.14	*Vibrio fortis*	2.09
Larvae (replicate 1)	A	No peaks found	0	No peaks found	0
Larvae (replicate 2)	A	*Vibrio fortis*	2.18	*Vibrio pelagius*	2.11
Larvae (replicate 2)	A	*Vibrio fortis*	2.28	*Vibrio pelagius*	2.23
Larvae (replicate 3)	A	*Vibrio fortis*	2.25	*Vibrio pelagius*	2.2
Larvae (replicate 3)	A	*Vibrio fortis*	2.26	*Vibrio pelagius*	2.23
Larvae (replicate 4)	A	*Vibrio fortis*	2.32	*Vibrio pelagius*	2.19
Larvae (replicate 4)	A	*Vibrio fortis*	2.2	*Vibrio pelagius*	2.19
Larvae (replicate 5)	B	*Vibrio fortis*	2.09	*Vibrio pelagius*	2.06
Larvae (replicate 5)	B	*Vibrio pelagius*	2.21	*Vibrio fortis*	2.17
Larvae (replicate 6)	B	*Vibrio fortis*	2.19	*Vibrio pelagius*	2.16
Larvae (replicate 6)	B	*Vibrio fortis*	2.24	*Vibrio pelagius*	2.21
Larvae (replicate 7)	B	*Vibrio fortis*	2.28	*Vibrio pelagius*	2.12
Larvae (replicate 7)	B	*Vibrio fortis*	1.84	*Vibrio pelagius*	1.76
Larvae (replicate 8)	B	*Vibrio fortis*	2.22	*Vibrio pelagius*	2.05
Larvae (replicate 8)	B	*Vibrio fortis*	2.35	*Vibrio pelagius*	2.29

Manipulation of the microbiome via diet (16S sequence data:)

Samples were taken throughout both the temperature and diet manipulation experiments with the intent to analyse *via* 16S sequencing. None of the samples collected throughout the temperature manipulation experiment provided insufficient levels of bacterial DNA and so are not included in sequence data. Similarly, only samples from the final time points of the diet manipulation experiment yielded sufficient quantities of bacterial DNA to be used for 16S sequencing (see Table 8).

Table 8: Showing all samples collected for the intended purpose of full-length 16S sequencing. Samples are shown according to the date on which they were collected, sample type (algae, larvae, water from larval culture or 24-hour aged water) and their experimental treatment group (*Tisochrysis galbana* vs *Tisochrysis galbana* + *Tetraselmis suecica*). Samples shown in yellow with Y indicate instances where DNA amplification was sufficient to proceed with full-length 16S sequencing. N denotes no, or insufficient amplification of bacterial DNA.

		Algae		Larvae								Water from larval culture (no larvae)								Aged water
		Tisochrysis galbana	*Tetraselmis suecica*	*Tisochrysis lutea*				*Tisochrysis galbana* + *Tetraselmis suecica*				*Tisochrysis lutea*				*Tisochrysis galbana* + *Tetraselmis suecica*				
Date collected	Age of larvae (days)	Rep 1	Rep 1	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1
16/06/2023	1	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
19/06/2023	4	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
22/06/2023	7	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
26/06/2023	11	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
29/06/2023	14	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N

In order to verify the 16S sequencing process, positive and negative control sequence data is summarised below in phylogenetic trees (Figure 15). Three positive control samples of *Streptococcus* bacteria resulted in a mean number of 119,436 reads (SD= 6840.5) (Figure 15a), whereas negative controls resulted in a mean number of 3,200 reads (SD= 2309.7) (Figure 15b).

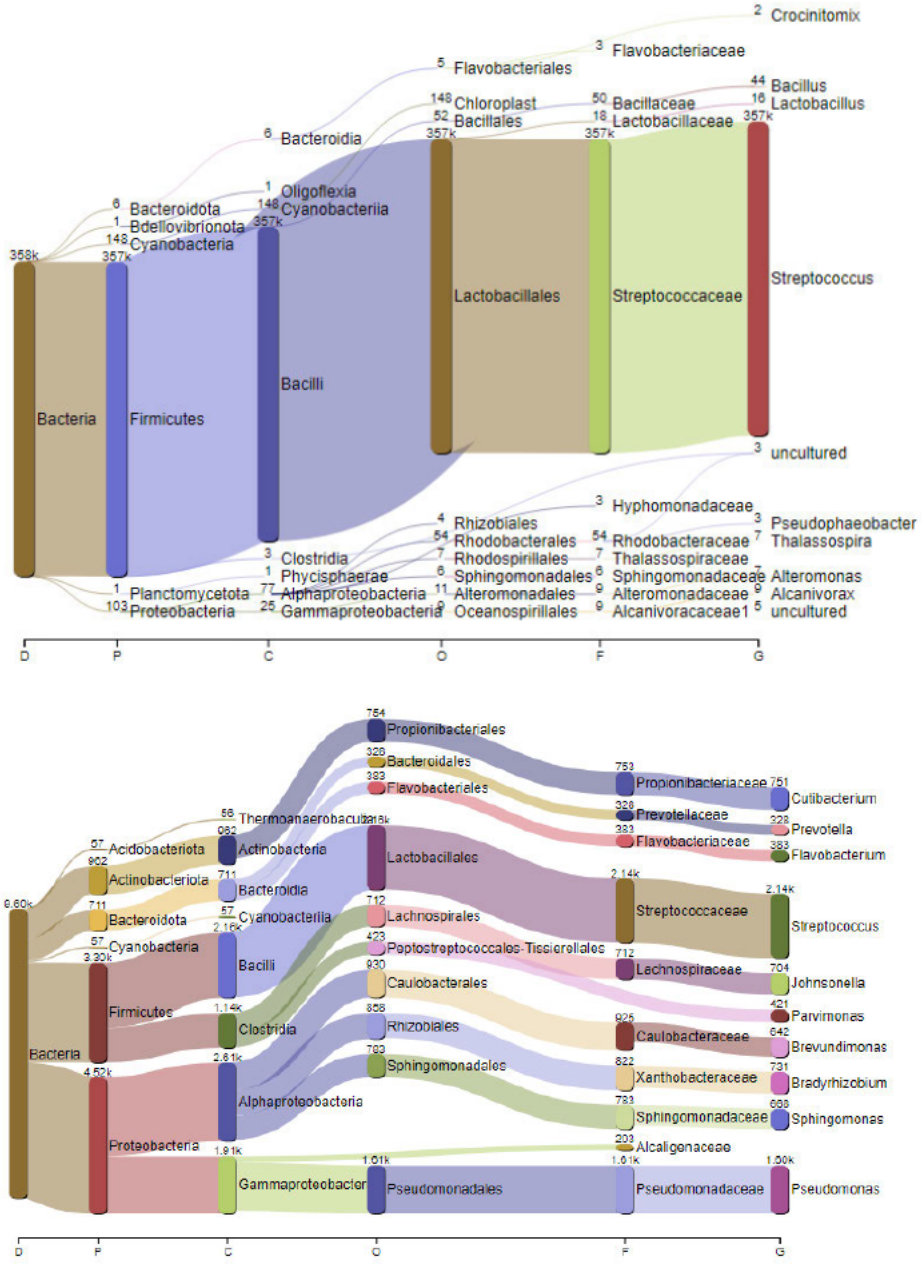


Figure 15: (a) Weighted phylogenetic tree of combined reads from three *Streptococcus* samples used for positive controls during the sequencing process. **(b)** Weighted phylogenetic tree of all combined reads from three nuclease-free water samples used as negative controls during the sequencing process.

Within the entire diet experiment dataset, a total of 557 OTUs were identified. The relative abundance of these OTUs is shown against its corresponding sample below (Figure 16). Algal samples collected prior to 29/06/2023 (annotated in green), show a lower relative abundance of the vast majority of OTUs identified in comparison to their overall mean. Little separation is seen between larvae and larval culture water samples, or between larval samples given differing diets (Figure 16). To investigate possible trends within the data further, this dataset was filtered and the relative abundance levels of 12 OTUs (which represented at least 10% of reads in at least one sample) shown (Figure 17). Similarly, algal samples showed a lower relative abundance of the 12 OTUs displayed. Samples of larvae and larval culture water showed few clear groupings.

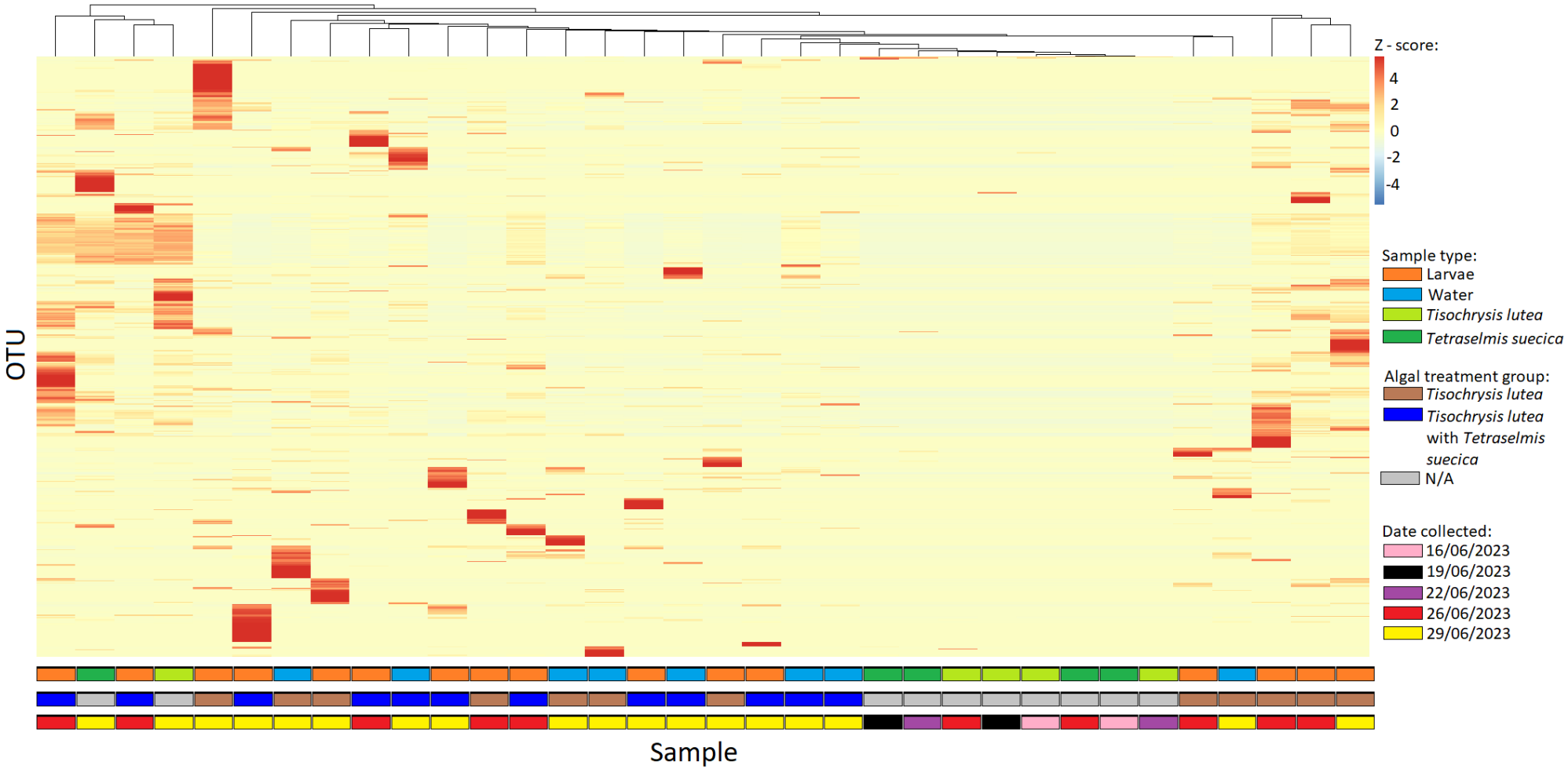


Figure 16: Relative abundance levels of all OTUs identified in water, larval and algal samples standardised for expression as Z-scores. Each sample is annotated according to its sample type, algal treatment group and collection date.

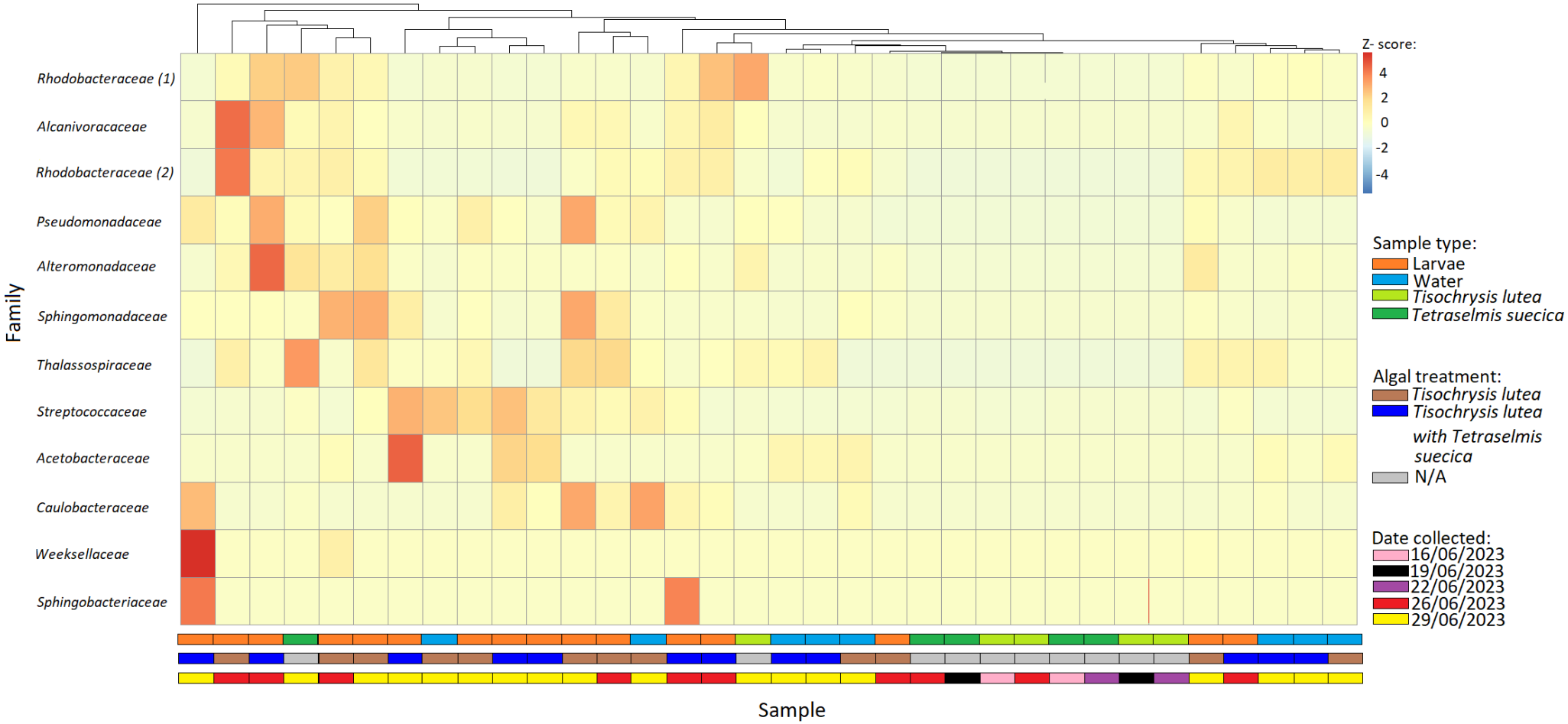


Figure 17: Relative abundance levels of OTUs that represent at least 10% of reads in at least one sample. Abundance levels are standardised for expression as Z- scores and each OTU organised according to its bacterial family. Each sample is annotated according to its sample type, algal treatment group and collection date.

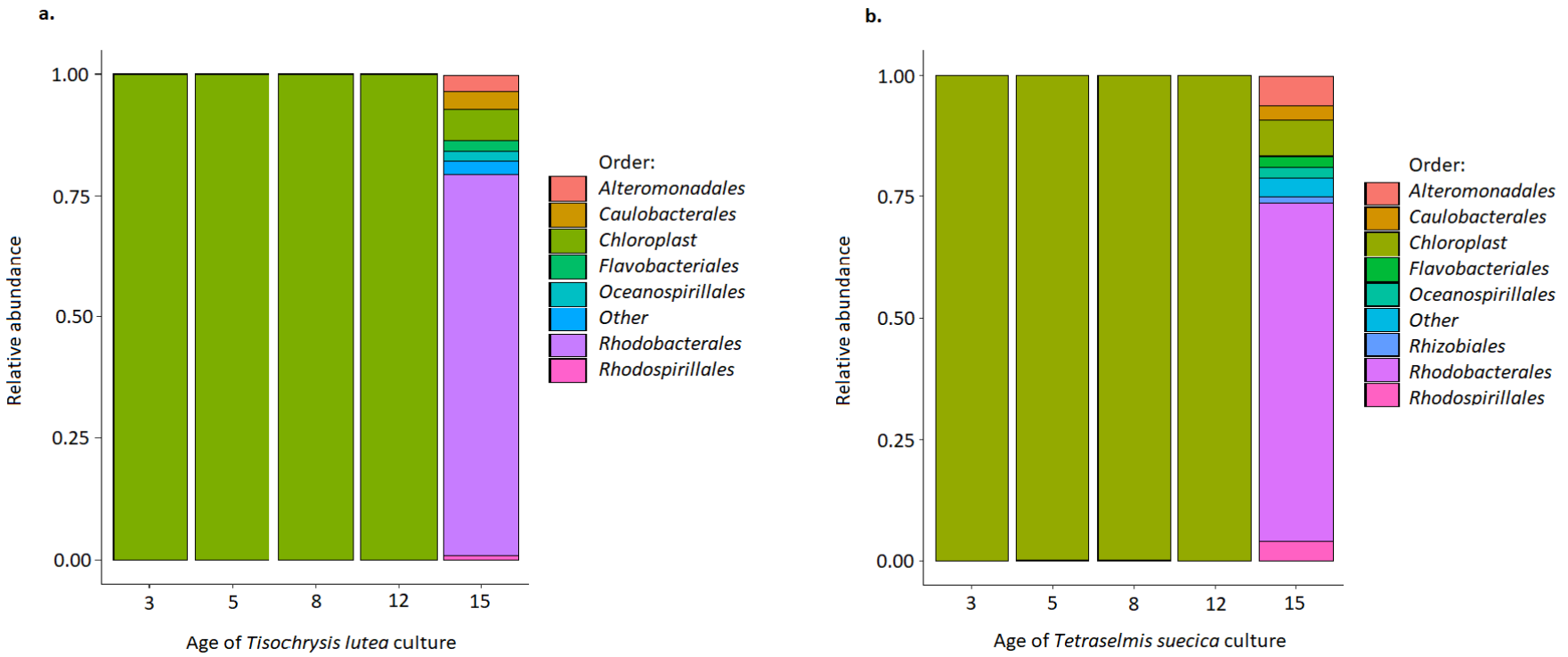


Figure 18: (a) Relative abundance of bacterial orders found in the same *Tisochrysis lutea* culture at 3, 5, 8, 12 and 15 days old. **(b)** Relative abundance of bacterial orders found in the same *Tetraselmis suecica* culture at 3, 5, 8, 12 and 15 days old. The majority of material amplified at 3,5,8 and 12 days is chloroplast from the algae.

Between 99 – 100% of reads from all algal samples collected between 3 – 12 days were identified as chloroplasts (Figure 18). In order to analyse microbial data appropriately, chloroplast reads were therefore excluded from all samples used in further analysis. To provide information about species richness between the two species of algae used, alpha diversity measures were calculated for 15-day old *Tisochrysis lutea* and *Tetraselmis suecica* (Figure 19). Chao1 and Shannon diversity measures for *Tisochrysis lutea* were reported at 260.00 and 2.070795 and 353.25 and 2.366028 for *Tetraselmis suecica*. With only one sample per group, statistical tests were not able to be completed to test for statistically significant levels of variation in alpha diversity indices between the two algal species.

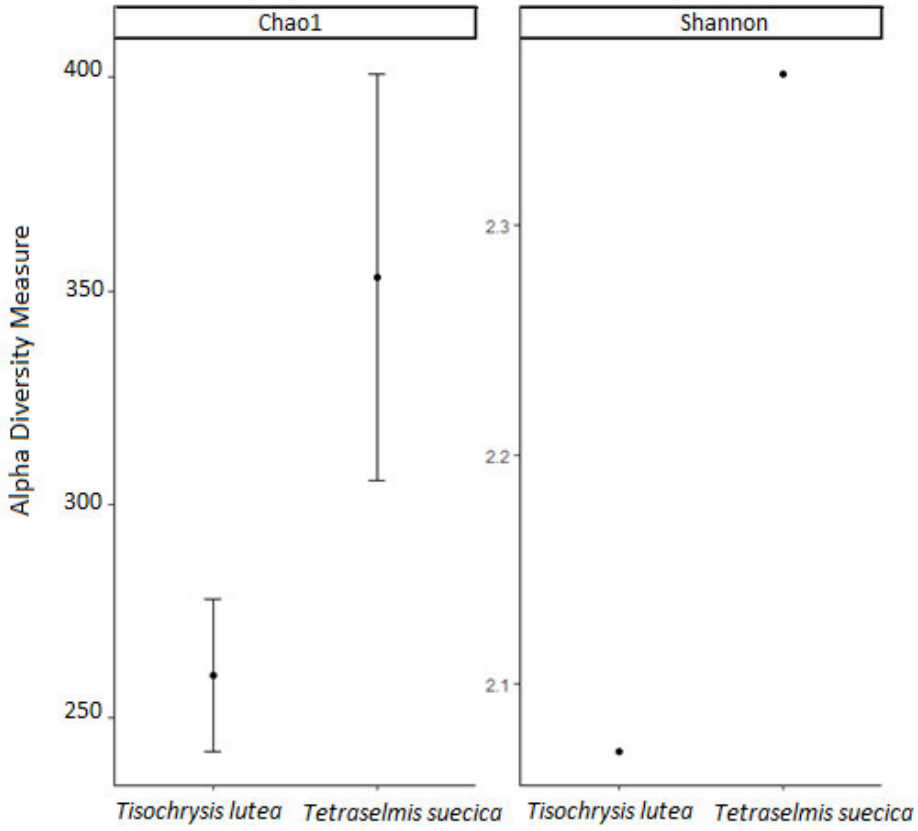


Figure 19: Chao 1 and Shannon alpha diversity measures from one *Tisochrysis lutea*, and one *Tetraselmis suecica* sample both at 15 days old (as previously shown in Figure 14). These alpha diversity measures were calculated after excluding chloroplast reads from both samples.

Microbial differences between larval samples across the two dietary treatment groups and ages (11-days old and 14-days old), were analysed via non-metric multidimensional scaling (NMDS); (Figure 20). Here, each data point represents one larval sample, which is coloured according it its dietary treatment group and age at collection. PERMANOVA statistical tests confirmed a statistically significant difference between larval samples of different dietary treatments ($p = 0.011$, $r^2 = 0.432$). Observed and Cha01 alpha diversity indices were also calculated with no statistical difference being found between treatment groups of 11-day old ($p= 0.9746$) or 14-day old ($p=0.4171$) larvae (Figure 21).

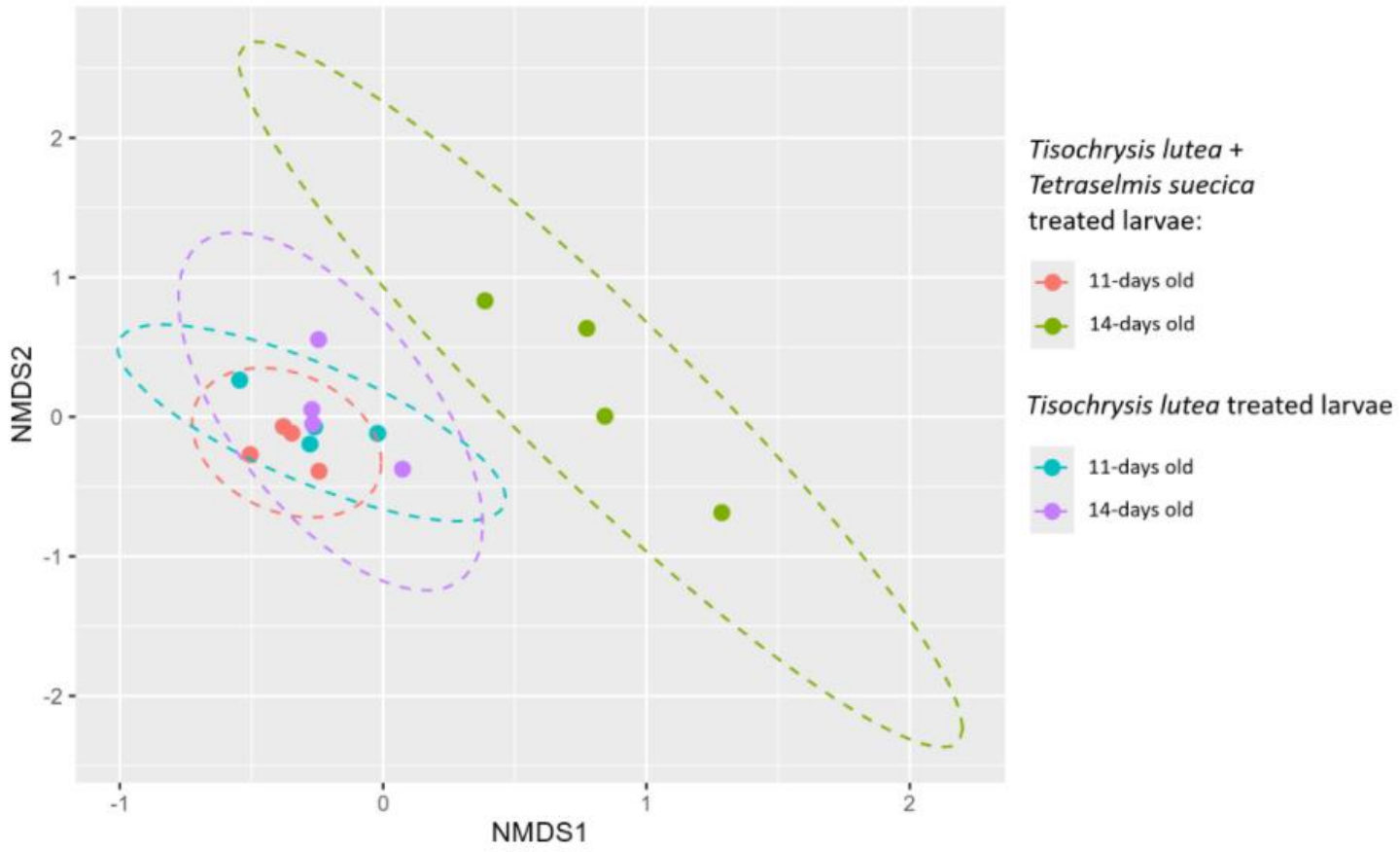


Figure 20: NMDS plot where each data point represents OTUs from individual larval samples. Data points are coloured according to age of larvae and their dietary treatment group. 11-day old larvae with *Tisochrysis lutea* (blue), 11-day old larvae with *Tisochrysis lutea* and *Tetraselmis suecica* (red), 14-day old larvae with *Tisochrysis lutea* (purple), 14-day old larvae with *Tisochrysis lutea* and *Tetraselmis suecica* (green). Ellipses around each sample group represents a 95% confidence interval.

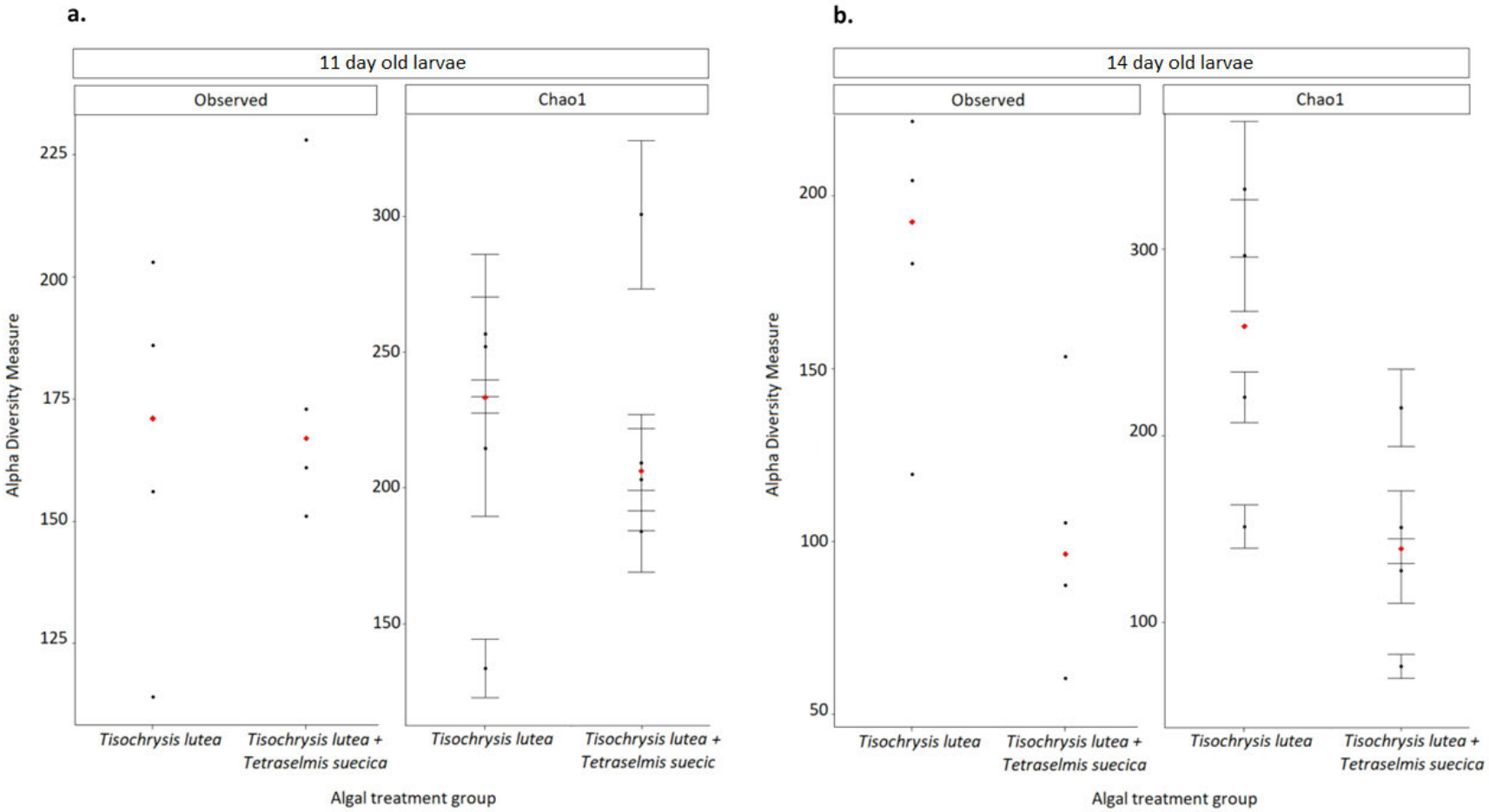

a.
11 day old larvae
Observed
Chao1
Alpha Diversity Measure
225
200
175
150
125
300
250
200
150
Tisochrysis lutea
Tisochrysis lutea + Tetraselmis suecica
Tisochrysis lutea
Tisochrysis lutea + Tetraselmis suecic
Algal treatment group
b.
14 day old larvae
Observed
Chao1
Alpha Diversity Measure
200
150
100
50
300
200
100
Tisochrysis lutea
Tisochrysis lutea + Tetraselmis suecica
Tisochrysis lutea
Tisochrysis lutea + Tetraselmis suecica
Algal treatment group

Figure 21: a Observed and Chao 1 alpha diversity indices of 11-day old larvae and under dietary treatment groups of *Tisochrysis lutea* or *Tisochrysis lutea* and *Tetraselmis suecica.* **b** Observed and Chao 1 alpha diversity indices of 14-day old larvae under dietary treatment groups of *Tisochrysis lutea* or *Tisochrysis lutea* and *Tetraselmis suecica.*

The microbial differences between larval samples (Figure 20) are therefore driven by abundances of certain taxa and not species richness (Figure 21). To visualise this relationship further, bar charts were created in order to show the bacterial composition of each sample, categorising each OTU at a class level. Bacterial compositions of four replicate tanks across the two treatment groups are shown below for both 10 and 13-day old larvae (Figures 22 and 23). The same twelve bacterial classes were detected in larvae of both age groups, with the majority of each larval sample being comprised of the *Alphaproteobacteria* class. Thirteen-day old larvae fed the experimental diet (*Tisochrysis lutea* with *Tetraselmis suecica*) had a higher relative abundance of *Bacilli* and *Actinobacteria* classes compared to those fed only *Tisochrysis lutea* (Figure 23).

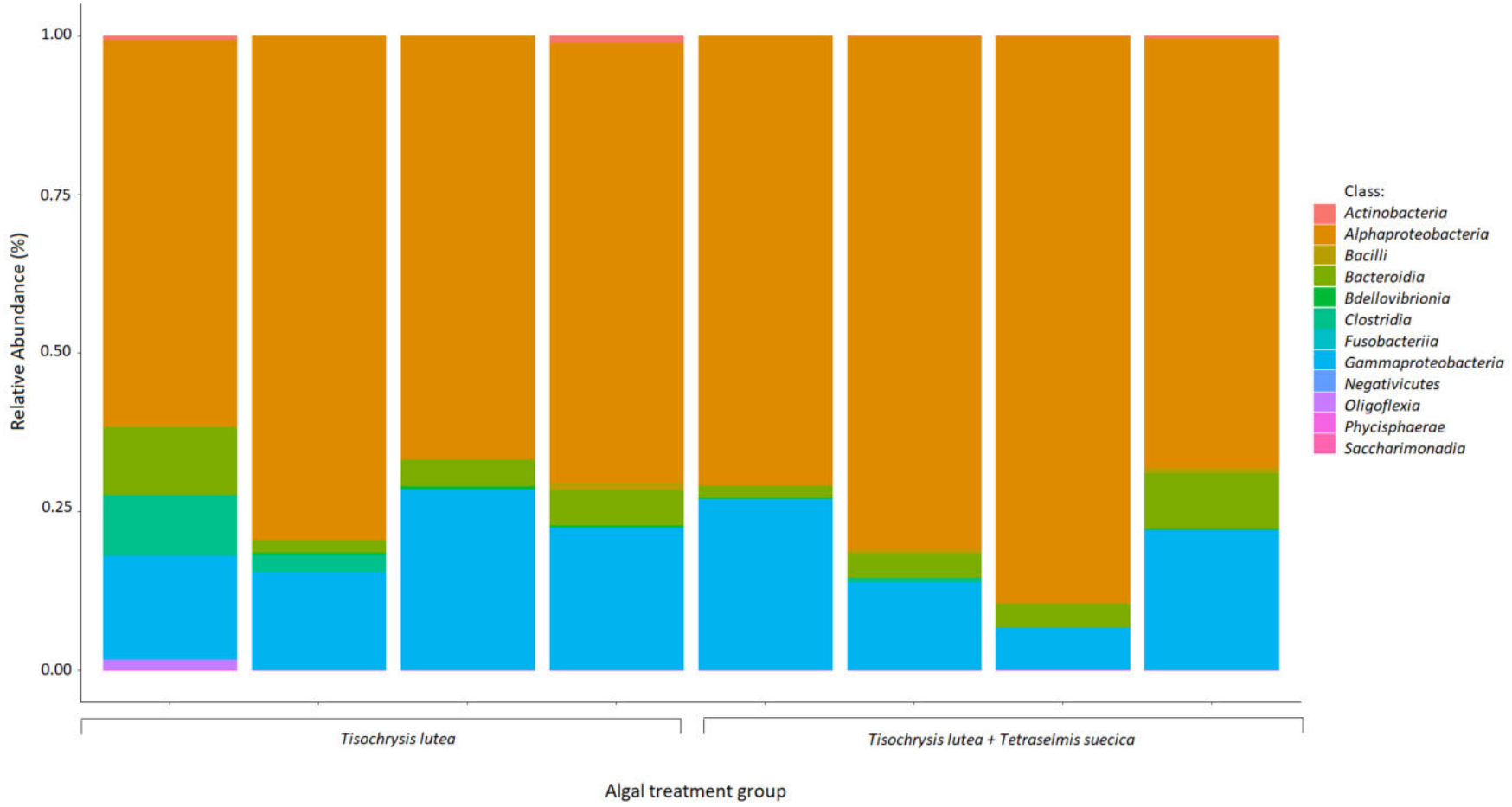


Figure 22: Relative abundance levels of the 12 bacterial classes found in 11-day old larvae. Each bar represents one experimental replicate across the two treatment groups (larvae fed *Tisochrysis lutea* or *Tisochrysis lutea* and *Tetraselmis suecica*).

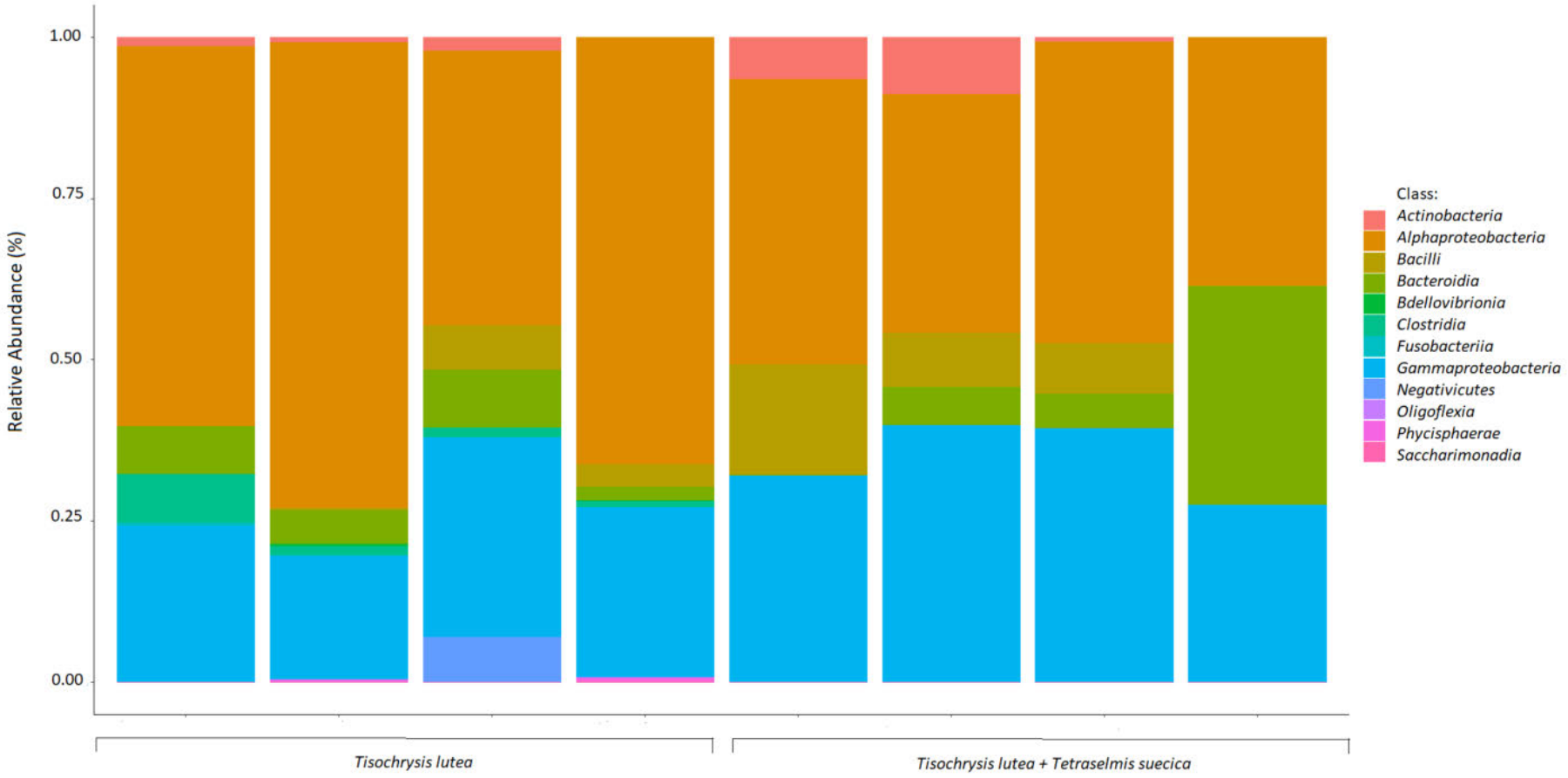


Figure 23: Relative abundance levels of the 12 bacterial classes found in 14-day old larvae. Each bar represents one experimental replicate across the two treatment groups (larvae fed *Tisochrysis lutea* or *Tisochrysis lutea* and *Tetraselmis suecica*).

When comparing the microbiomes of the two different algal species at 15-days old, 17 OTUs were found to be present in cultures of *Tetraselmis suecica*, but not *Tisochrysis lutea.* The taxonomic classifications of these 17 OTUs and their relative abundances within *Tetraselmis suecica* are listed below (Table 9).

Table 9: Taxonomic classification and relative abundance levels of 17 OTUs found to be unique to the 15-day old culture of *Tetraselmis suecica.*

Order	Family	Genus	Relative abundance
Rhodobacterales	Rhodobacteraceae	*Antarctobacter*	1.13286×10^{-4}
Rhizobiales	Xanthobacteraceae	NA	1.75079×10^{-4}
Parvibaculales	Parvibaculaceae	*Parvibaculum*	2.05975×10^{-4}
Burkholderiales	Neisseriaceae	NA	1.6478×10^{-4}
Burkholderiales	Neisseriaceae	*Neisseria*	5.14938×10^{-4}
Burkholderiales	Alcaligenaceae	NA	1.44183×10^{-4}
Burkholderiales	Alcaligenaceae	*Achromobacter*	1.13286×10^{-4}
Bacteroidales	Porphyromonadaceae	*Porphyromonas*	1.071072×10^{-3}
Staphylococcales	NA	NA	1.02988×10^{-4}
Staphylococcales	Gemellaceae	*Gemella*	2.966045×10^{-3}
Bacillales	Bacillaceae	*Bacillus*	1.02988×10^{-4}
Lachnospirales	Lachnospiraceae	NA	4.8402×10^{-4}
Lachnospirales	Lachnospiraceae	*Vallitalea*	2.667381×10^{-3}
Lachnospirales	Lachnospiraceae	*uncultured*	1.54482×10^{-4}
Bacteriovoracales	Bacteriovoracaceae	*Halobacteriovorax*	1.483022×10^{-3}
Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	6.48822×10^{-4}
Micrococcales	Micrococcaceae	*Rothia*	1.85378×10^{-4}

Of the 17 OTUs found in *Tetraselmis suecica* and not *Tisochrysis lutea* cultures, five (belonging to genera of *Antarcobacter*, *Halobacteriovorax*, *Porphyromonas*, *Neisseria* and *Achromobacter*), were also detected in larval samples. The *Neisseria* and *Porphyomonas* genera were found in larvae fed with *Tetraselmis suecica,* in one of the four replicate tanks for this treatment group (Figure 24).

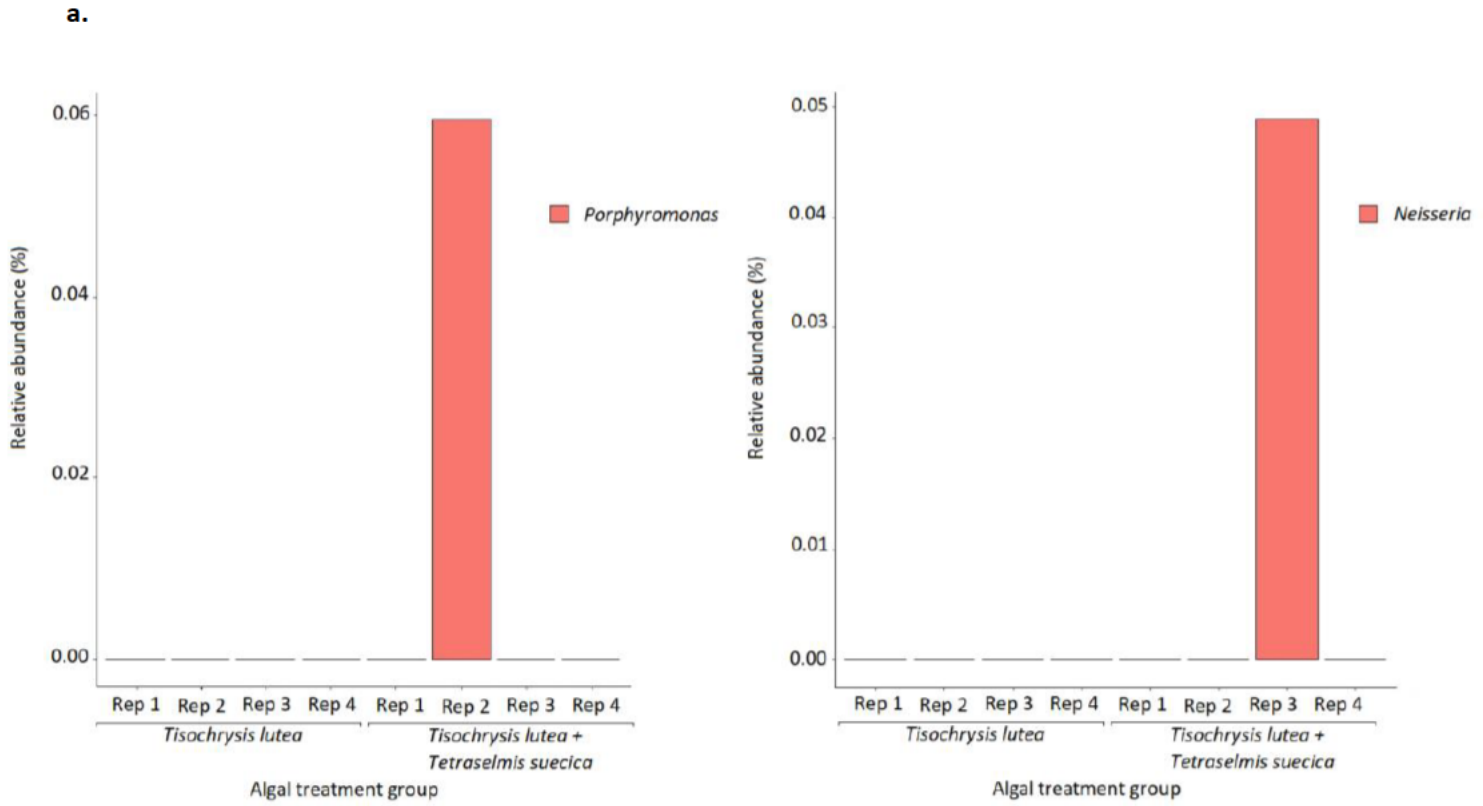


Figure 24: Relative abundance levels of two OTUs identified as belonging to the **(a)** *Porphyomonas* and **(b)** *Neisseria* genera across larvae fed diets of *Tisochrysis lutea* vs *Tisochrysis lutea* and *Tetraselmis suecica* (four replicate tanks per treatment group).

To compare the overlap of OTUs between larvae and algal samples, Venn diagrams were drawn incorporating samples collected under the two dietary treatments (Figure 25). In order to display only reproducible results, OTUs shown within larval and water samples were present in all four replicate tanks. Within *Tisochrysis lutea* treated larvae (Figure 25a), 43 OTUs were found to be common between all four replicate tanks of larvae at both ages, water and 15-day old *Tisochrysis lutea*. In comparison, fewer OTUs (10), were found to be common between all four replicate tanks of larvae at both ages, water and 15-day old *Tetraselmis suecica* (Figure 25b).

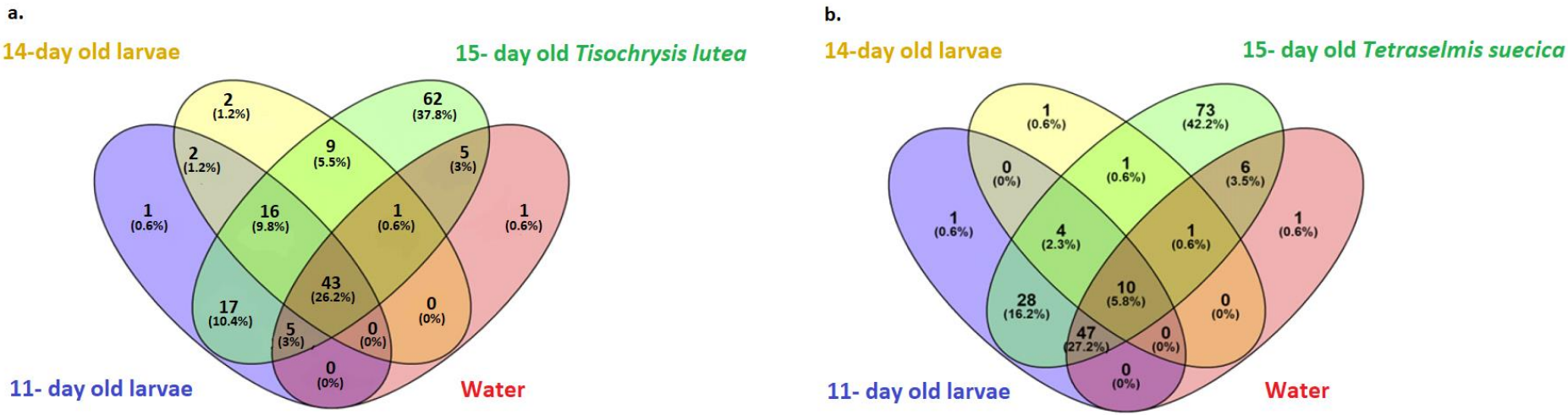


Figure 25: a Venn diagram representing OTUs detected in 15- day old *Tisochrysis lutea* (collected on 29/06/2023), larval culture water (collected on 29/06/2023), 11 -day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023) reared under a diet of *Tisochrysis lutea* only. OTUs shown in larval samples were present in each four replicate tanks. **b** Venn diagram representing OTUs detected in 15- day old *Tisochrysis lutea* (collected on 29/06/2023), larval culture water (collected on 29/06/2023), 11 -day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023) reared under a diet of *Tisochrysis lutea* and *Tetraselmis suecica*. OTUs shown in larval samples were present in each four replicate tanks.

The relative abundance of OTUs shown in Figure 25a across all samples under *Tisochrysis lutea* treatment are shown in Figure 26 below. An OTU identified as belonging to the *Rhodobacteraceae* family is highlighted as particularly prominent in all samples, with the highest relative abundance in *Tisochrysis lutea* (Figure 26).

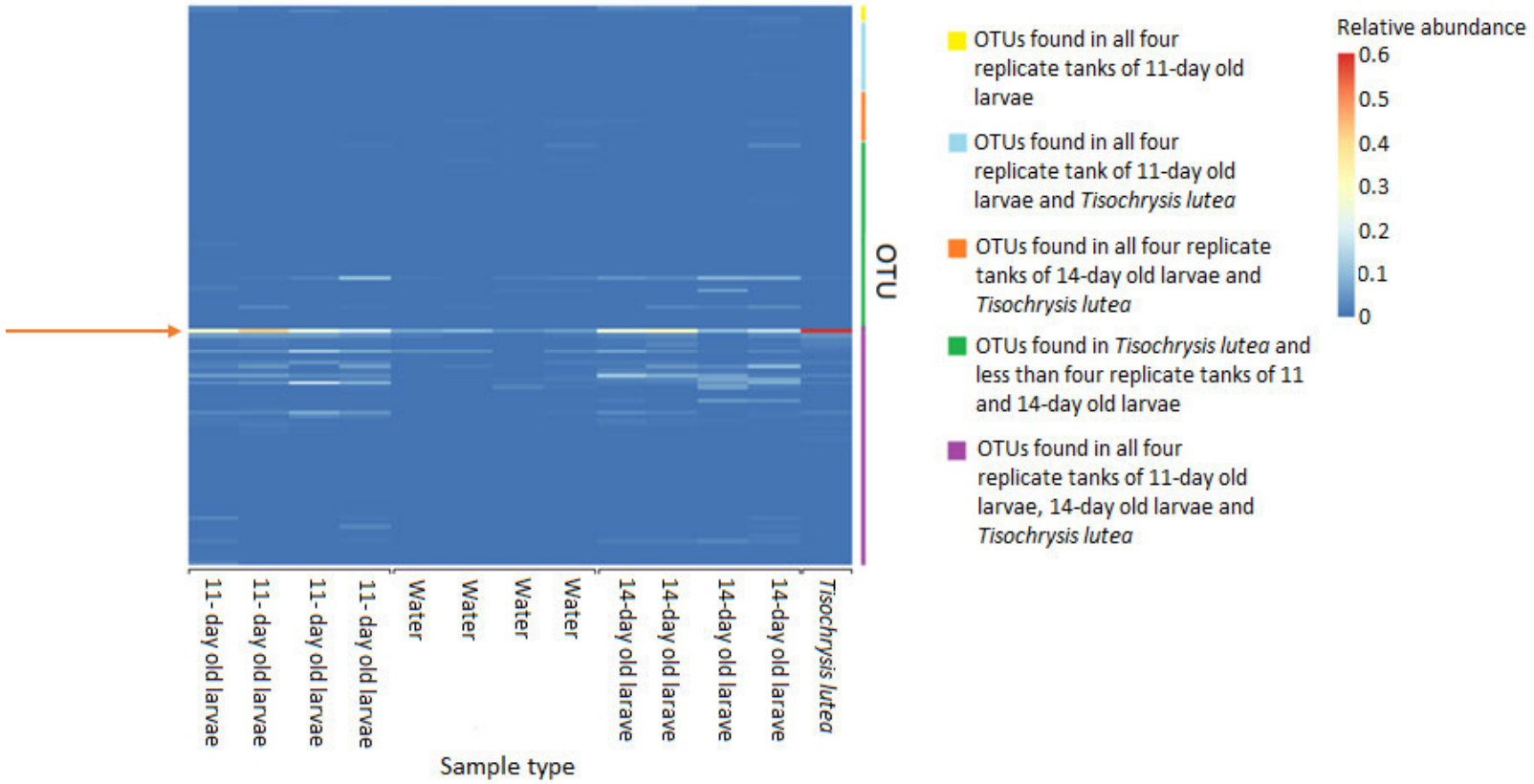


Figure 26: Heatmap showing the distribution of OTUs across 15-day old *Tisochrysis lutea (collected on 29/06/2023),* 11-day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023), reared on a diet of *Tisochrysis lutea*, and water samples (collected on 29/06/2023). The abundance of an OTU belonging to the *Rhodobacteraceae* family of an unknown genus and species is highlighted by an orange arrow.

In order to investigate other taxa of importance, the Rhodobacteraceae OTU previously shown in Figure 26 was removed (Figure 27). Here, OTUs belonging to the *Alcanivorax* and *Tabrizicola* genera are identified as being particularly abundant in 11 and 14-day old larval samples.

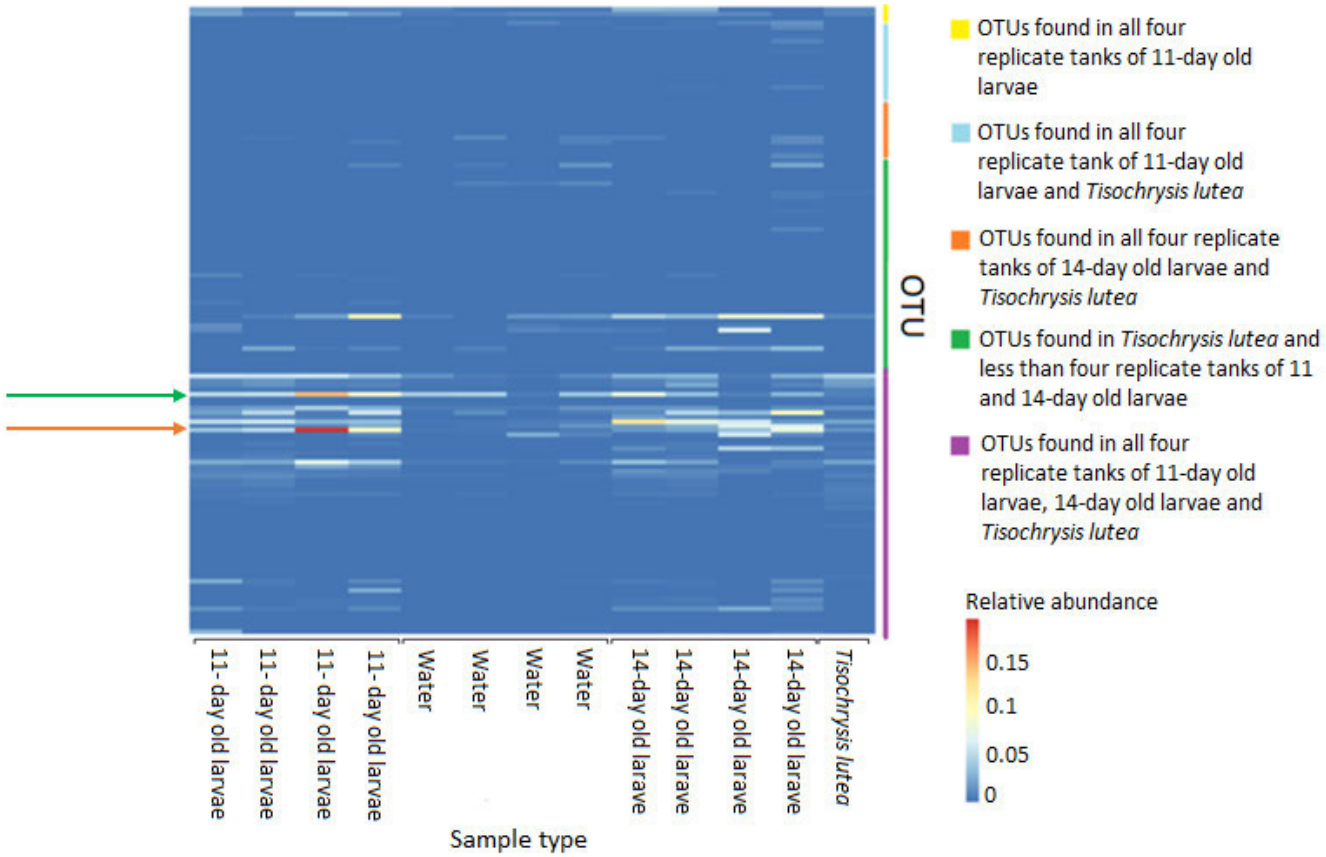


Figure 27: Heatmap showing the distribution of OTUs across 15- day old *Tisochrysis lutea* (collected on 29/06/2023), 11-day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023) reared under a diet of *Tisochrysis lutea*, and water samples (collected on 29/06/2023). OTUs belonging to the *Alcanivorax* and *Tabrizicola* genera are also highlighted by orange and green arrows.

The relative abundance of OTUs shown in Figure 25b across all samples under *Tetraselmis suecica* treatment are shown in Figure 28 below. The same OTU as is shown in Figure 26 was also identified in this treatment group (Figure 28), being most abundant in the algal and 11-day old larval samples.

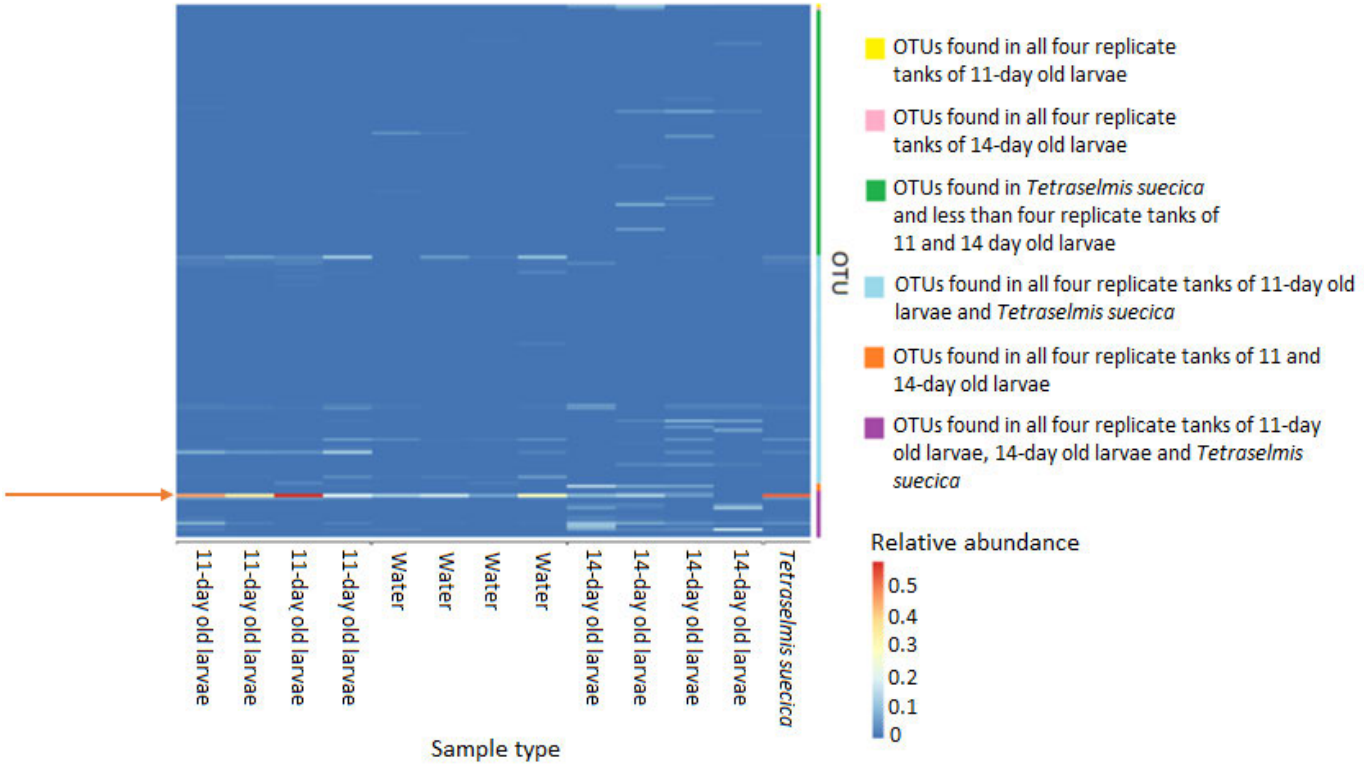


Figure 28: Heatmap showing the distribution of OTUs across 15- day old *Tetraselmis suecica (collected on 29/06/2023),* 11-day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023) reared under a diet of *Tisochrysis lutea and Tetraselmis suecica*, and water samples (collected on 29/06/2023). The abundance of an OTU belonging to the *Rhodobacteraceae* family of an unknown genus and species is highlighted by an orange arrow.

After removing the OTU highlighted in Figure 28, OTUs with high abundances in 11 and 14-day old larvae are highlighted and have been identified as belonging to the *Pseudophaeobacter* and *Pseudomonas* genera. The relative abundance of *Pseudophaeobacter* was highest in 11-day old larvae and water samples, whereas *Pseudomonas* was more relatively abundance in 14-day old larvae.

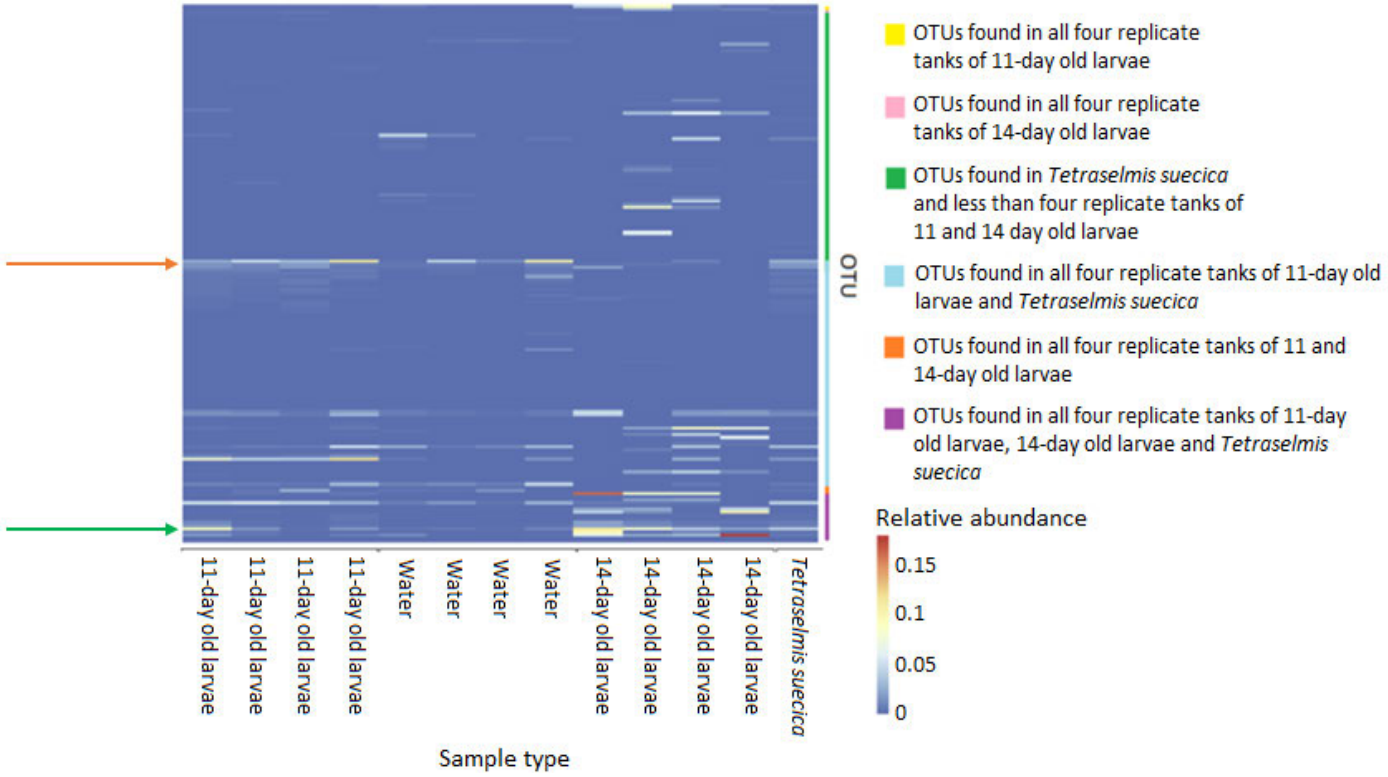


Figure 29: Heatmap showing the distribution of OTUs across 15- day old *Tetraselmis suecica (collected on 29/06/2023),* 11-day old larvae and 14-day old larvae (collected on 26/06/2023 and 29/06/2023) reared under a diet of *Tisochrysis lutea and Tetraselmis suecica*, and water samples (collected on 29/06/2023). OTUs belonging to the *Pseudophaeobacter* and *Pseudomonas* genera are also highlighted by orange and green arrows respectively.

In order to compare microbial profiles of 14-day old larvae and their corresponding water samples, NMDS analysis was first conducted and is shown below alongside algal samples (Figure 30). A PERMANOVA test confirmed differences between larval, water and algal samples to be statistically significant ($p = 0.0006$, $r^2 = 0.345$).

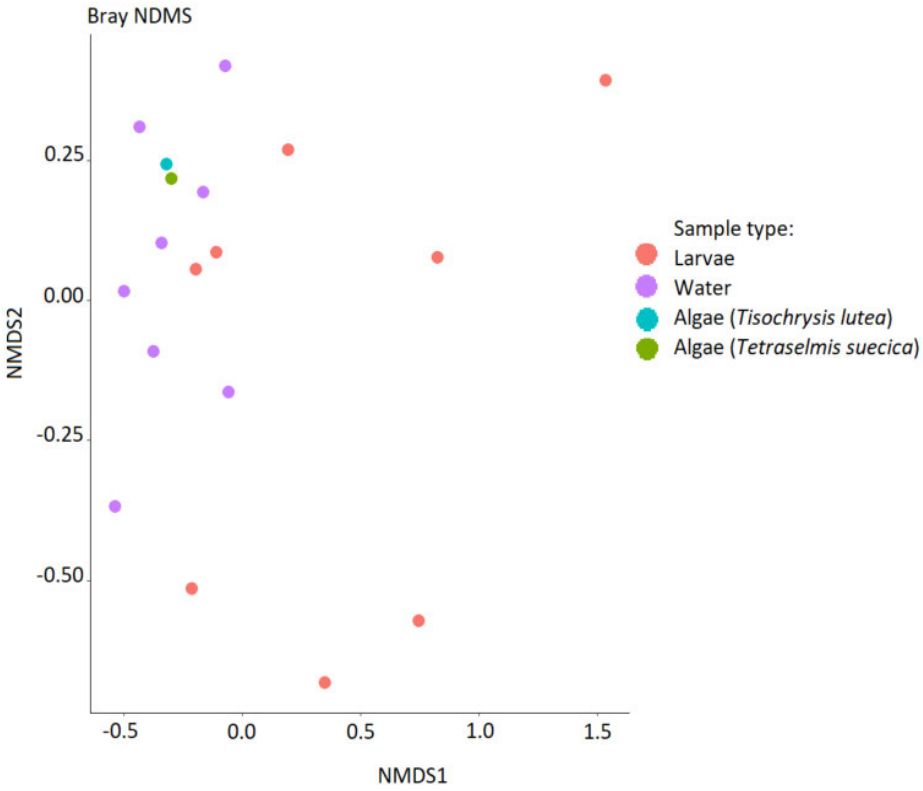


Figure 30: NMDS analysis showing water (X8, purple), larval (X8, red), *Tisochrysis lutea* (X1, blue) and *Tetraselmis suecica* (X1, green) samples collected on 29/06/2023.

The species richness between these different sample types (alpha diversity) was also calculated, reporting average diversity measures for larval samples to be 131 (Observed) and 169.2 (Chao1). Alpha diversity measures in the water are reported to be slightly higher at 140 (Observed) and 187.5 (Chao1), (Figure 31). A Kruskal-Wallis statistical test confirmed there to be no statistical differences in alpha diversity indices between these two different sample types (p=0.3827).

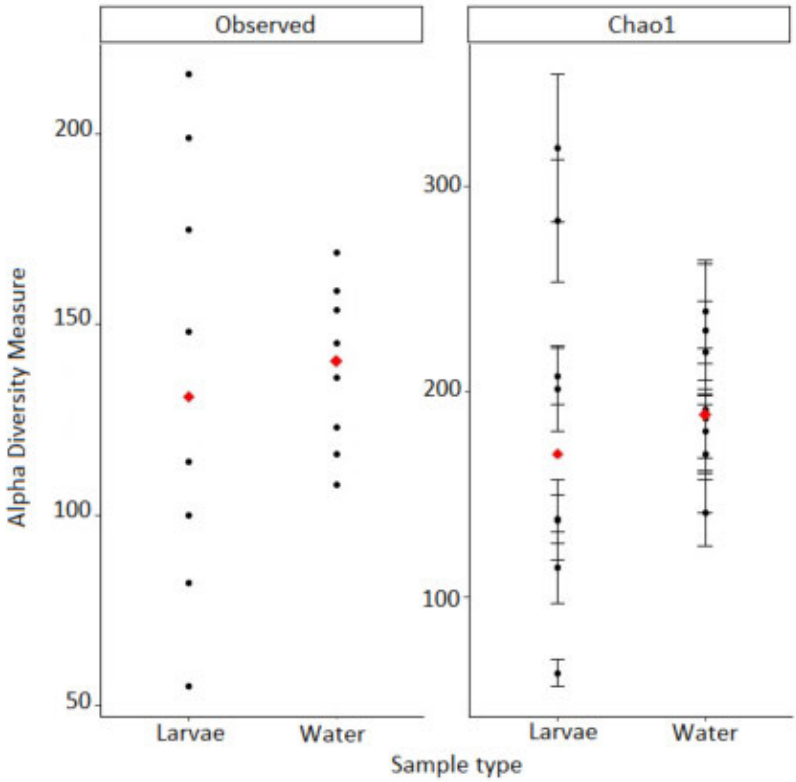


Figure 31: Observed and Chao1 alpha diversity indices for eight 14-day old larval samples and their corresponding water samples collected on 29/06/2023. Mean alpha diversity values for each sample type are shown in red.

Discussion:

Insufficient 16S rRNA amplification from select samples:

The initial experimental design included 16S rRNA sequencing across all samples collected, enabling bacterial ID and abundance data to be calculated across both experiments. However, post 16S PCR, two thirds of the original sample set (e.g., Table 7) yielded insufficient levels of bacterial 16S amplicon to proceed with sequencing. The sequencing process included three positive controls of *Streptococcus* which were all successfully amplified (Figure 11a). The lack of bacterial DNA captured from select samples is therefore not thought to be due to flaws in the DNA extraction or sequencing process, but due to the very low abundance of bacteria or any genetic material in the original samples.

The low bacterial abundance seen across many samples may have resulted from a number of different factors. On a broad scale, the experimental facilities provided a more sterile environment in comparison to commercial hatcheries. More specifically, in the case of temperature manipulation, samples of larvae were not condensed on a sieve prior to sampling. It is therefore possible that too few larvae were collected during this sampling process and with an insufficient biomass per sample, a microbial profile could not be captured. In order to increase the possibility of capturing the larval microbiome, future manipulation experiments would benefit from housing larvae in larger volumes and/or condensing the number of larvae collected in each sample.

Although diet manipulation experiments used larger volumes of larval cultures, sufficient levels of bacterial DNA could not be amplified from larvae at 1-, 4- or 7-days post fertilisation. This may provide evidence that younger larvae do not have a strong bacterial signature, with OTUs obtained from sources such as water, gametes, air and food taking time to become established within the host. There is currently a lack of literature reporting microbial data in younger larvae, with Stevick et al, 2019 and Vignier et al, 2021 capturing the microbiomes of the youngest larvae at 5- and 7-days post fertilisation. These findings highlight the challenges of capturing robust microbiome data from early-stage larvae and low-biomass samples, emphasizing the need for optimised sampling strategies and careful consideration of larval developmental stage in future experimental designs.

Manipulation of the microbiome via temperature:

The optimal temperature for rearing Pacific oyster larvae (between 25-27°C) has been well described (Meng et al, 2021), resulting in an increased growth rate and in turn, faster production of seedstock. However, the effect that operating at these higher temperatures has on the larval microbiome remains understudied. Past studies have reported microbiome dysbiosis in adult oyster haemolymph in response to increased water temperatures intended to simulate climate stressor events (Lokmer and Wegner, 2015). The effects of temperature change on the larval microbiome within the context of routine hatchery practices however remains understudied. We therefore aimed to collect both culturable and non-culturable microbial data from oyster larvae reared at 20°C and 25°C, to investigate whether a higher rearing temperature may select for a less stable larval microbiome, thus encouraging disease mortality events.

No distinct differences in the morphologies of bacterial colonies grown from samples collected throughout this experiment were observed, with all appearing as small, flat, white colonies after 48 hours (Figure 9). The abundance of culturable bacteria obtained from each experimental replicate increased throughout the experiment as larvae progressed from 4 (average of 19 CFU/ml) to 15 days old (average of 1013.75 CFU/ml), (Figure 8). This trend however is also observed in colonies collected from negative control beakers (containing no larvae). It is therefore expected that the CFU data captured during this experiment is more representative of the larval culture water and is not evidence of larvae acquiring a microbial signature with age. In order to capture microbial data that is distinct between the larvae and their culture water, it is recommended that future experiments use cultures of greater volumes where larvae can be condensed prior to sampling.

In agreement with current literature (Meng et al, 2021), a larval rearing temperature of 25°C resulted in significantly larger larvae ($p = 0.0082$), than those reared at 20°C. However, without 16S rRNA sequence data it is not possible to identify taxonomic differences in the microbiomes of larvae reared at these two different temperatures. In order to ensure that the full extent of 16S sequence data can be captured from further research, future

experiments would benefit from prolonged time frames sampling larvae continuously up to settlement (approximately 20 days post fertilisation).

As expected, elevated rearing temperature significantly increased larval growth. However, the absence of 16S rRNA data prevented clear conclusions about temperature-driven effects on the larval microbiome. This highlights the need for improved methodologies and extended sampling periods in future research.

* * *

Growth rate, CFU/mL, and 16S rRNA analysis of algal cultures:

Cell densities of algal cultures were recorded periodically between three and sixteen days of growth (Figure 11a). This allowed algal cultures to be sub-cultured at appropriate time points as well as an opportunity for visual health checks. Figure 11a shows an overall increasing cell density within cultures of both *Tetraselmis suecica* and *Tisochrysis lutea* over time. This is positive in assuring that larvae were fed cultures of fed live, healthy microalgae – an essential criterion of the study.

Samples of three- and five-day old *Tetraselmis suecica* and *Tisochrysis lutea* both resulted in zero CFU/mL, likely a result of beginning from axenic cultures which are often assumed to be sterile. CFUs were however captured from both algal cultures of eight days old, suggesting the introduction of bacteria. This would most likely have occurred through the sub-culturing process, when the culture vessels were opened for sampling and transfer to a larger volume. In a less sterile hatchery environment, it is likely that bacteria may be introduced into algal cultures in a similar way – highlighting a possible transfer route of harmful bacteria to larvae.

In order to investigate the influence that differing dietary treatments may have had upon larval microbiomes, it is first key to consider the microbial compositions of the algal samples themselves. Future studies would benefit from increased sample sizes to allow for statistical testing of differences in species richness between the two species. The microbial composition of both species was comprised of over 65% Rhodobacterales (Figure 18). This is a diverse

order of bacteria, commonly found within the marine environment, with the Rhodobacteraceae family being the most widely distributed bacteria in the marine environment (Pohlner et al, 2019). Despite this common order, microbial differences between algal samples were also reported. Most notably, *Tetraselmis suecica* had a higher abundance of the Rhodospirillales and Alteromonadales orders in comparison to *Tisochrysis lutea* (Figure 18). These bacterial orders have also been previously reported within the marine environment and adult oysters (Offret et al, 2020) and larvae (Asmani et al, 2016). This divergence in bacterial communities associated with the two algal species may have become more pronounced if algal cultures been maintained over a longer period.

After discarding reads from OTUs with a relative abundance less than 0.0001, 17 OTUs were found in *Tetraselmis suecica*, which were not present in *Tisochrysis lutea* (see Table 9). Only two of these 17 OTUs (belonging to the *Porphyromonas* and *Neisseria* genera) were also detected in 14-day old larvae in the *Tetraselmis suecica* treatment group (Figures 24a and 24b). Most importantly, the presence of these OTUs is not consistent across replicate tanks. Therefore, whilst these OTUs do appear to have been passed onto larvae in some cases, this was not a reproducible effect. This inconsistent transfer of specific OTUs to larvae suggests that dietary microbes may not reliably shape the larval microbiome without prolonged exposure.

* * *

Manipulation of the microbiome via diet:

Larvae given Tetraselmis suecica had an increased growth rate:

The production of live microalgae comes at high expense to commercial hatcheries accounting for an estimated one third of expenditure in the rearing of larvae into spat (Benemann, 1992). More economic methods of feeding via dehydrated algae or algal pastes have been investigated, but deemed unfavourable due to their insufficient shelf lives and associated reductions in larval growth (Robert et al, 2001). Live microalgae are therefore routinely produced by hatcheries with species of the *Tisochrysis* genera being commonly used to rear *Crassostrea gigas* as well as *Ostrea edulis* (European flat oyster) and *Saccostrea glomerata* (Sydney Rock oyster), (Leal et al, 2021; Robert et al, 2017). Past literature has hypothesised that mixed feeds which combine algal species are beneficial for larval production (da Costa et al, 2023; Rico-Villa et al, 2006). For example, although *Tetraselmis suecica* cells are known to be too large to be ingested by larvae younger than 12-days old, their inclusion into algal feeds with *Tisochrysis* proved beneficial to the growth rate of *Ostrea edulis* larvae between zero and eight days old (Helm, 1977). Using a different oyster species, a similar result was replicated here (Figure 12), with 14-day old larvae given *Tetraselmis suecica* as well as *Tisochrysis lutea* being larger ($p = 0.0001$), than those fed only *Tisochrysis lutea*. This difference between treatment groups is an important finding, with larval growth rates being a key criterion in the ability for hatcheries to reliably and consistently produce seedstock. Whilst this finding suggests that supplementing larval diets with Tetraselmis suecica can significantly enhance growth, the underlying mechanism e.g. exposure to different microbes, alteration of water chemistry or water quality) remains to be understood.

Culture dependent analysis of larval samples:

Whilst larval microbiome studies commonly report bacterial profiles via 16S sequencing, few combine these results with CFU data. CFU data is however important in order to investigate the viable bacterial load of the microbiome. At 14-days old, a higher abundance of culturable bacteria was obtained from larval samples compared to their corresponding water samples, suggesting it is the larvae themselves that harbour these bacteria. As this relationship was not replicated in samples of 3-day old larvae, this could also suggest that as larvae mature, their

microbiomes become more established or are able to retain more bacteria from their environment. This result may however also have been seen as a result of older larvae giving a greater biomass per sample and therefore more CFUs overall.

Data obtained from MALDI-TOF analysis (Table 7), matched CFUs most closely to either *Vibrio fortis* or its closest relative *Vibrio pelagius*. Previously, *V. fortis* has been isolated from a range of different marine environments including diseased *C. gigas larvae* (Romalde et al, 2014). Green et al, 2019 points to the potential pathogenicity of this species by reporting increased levels of *V. fortis* in *C. gigas* spat placed under thermal stress. In comparison to sequence data, an OTU belonging to the *Vibrio* genera was also detected within 14-day old larval samples via 16S sequencing. This OTU was detected in all four replicate tanks of larvae treated with *Tetraselmis suecica,* but just one the replicate tanks of larvae given the control diet. Had larvae been maintained until metamorphosis, *in vivo* effects of differing *V. fortis* abundance levels between treatment groups may have been observed. Possible sources of *Vibrio* OTUs in may include the surrounding seawater, algal feed or parental gametes. Overall, larvae at 14-days old had a greater culturable bacterial load, suggesting a more established or retained microbiome with age. The presence of OTUs belonging to the *Vibrio* genera were detected through both MALDI-TOF and 16S rRNA sequencing, with no negative impact to larval health observed.

* * *

16S rRNA sequence data from larval samples:

Larvae reared throughout this experiment appeared motile, with very few abnormalities or empty shells observed (Figures 8 and 13). This study is consequently not expected to have been affected by a disease event, and the microbial profiles shown are representative of those from healthy oyster larvae. The red colourations within photographed larvae were actively feeding (Figures 8 and 13), meaning that the effects of diet treatments could be appropriately tested.

The microbial compositions of *Tetraselmis suecica* treated 14-day old larvae were the least similar those of larvae at any other age or dietary treatment group (Figure 20). As previously discussed, the presence of an OTU in *Tetraselmis suecica* did not guarantee its transfer to the larvae. The microbial differentiation of this larval group (Figure 20) may alternatively be a result of random variation in microbial community, reflecting natural stochasticity rather than a true biological or treatment-driven effect. OTUs associated with larvae collected at 11-days old also appear to be grouped more closely together compared to those collected at 14-days old. This may suggest that bacterial profiles may become more varied within larvae as they age. Again, prolonging sample collection to incorporate oyster larvae up to the metamorphosis stage would be required in order to understand this relationship further.

NMDS analysis showed that differing sample types (culture water, larvae, *Tisochrysis lutea* and *Tetraselmis suecica*) accounted for 34% of the statistically significant ($p = 0.0006$) microbial variations shown in Figure 30. Figure 31 concluded that these statistical differences were not due to differences in species richness ($p = 0.3827$), but by differences in the abundance of certain taxa – most notably an increased abundance of Gammaproteobacter in larval samples. Similar findings have been previously reported between *Crassostrea virginica* larvae (the Eastern oyster) and its culture water, reporting statistical differences in overall richness but not diversity between the two (Arfken et al, 2021). This distinction between microbiomes is key information in determining the source of the larval microbiome. Detecting the source of the larval microbiome has great potential to positively impact the control of hatchery disease events, making them easier to detect and treat. Although filter feeders have great potential to be influenced by environmental microbiomes, these results suggest that bacteria present in larval culture water are not proportionately transferred to larvae.

Can the source of larval OTUs be identified?

A total of 43 OTUs were shared between larval water, *Tisochrysis lutea*, 11 and 14-day old larvae (Figure 25a). This indicates that a wide range of bacterial taxa are shared between these four different sample types. Very few OTUs were found to be distinct to larval or water samples with no more than two found exclusively in these groups (Figure 25a). Therefore, despite finding taxa common between larvae and algae, the presence of an OTU in *Tisochrysis lutea*, did not guarantee its transfer to larvae. However, nearly all the bacteria present in all

larval and water samples were also present in the algae. These contrasting results suggest that either the algae is the source of bacteria in the water and larval samples, or that there is a common source of contamination for all samples in addition to the algae.

Comparing this information with *Tetraselmis suecica* treated samples (Figure 25b), only 10 OTUs were found common to larval samples of both ages, *Tetraselmis suecica* and larval water samples (Figure 25b). Figure 25b also shows 89 common OTUs between the *Tetraselmis suecica* and 11-day old larvae compared to just 16 shared between *Tetraselmis suecica* and 14-day old larvae. Since the 10-day old larvae had not been exposed to the *Tetraselmis suecica* shown in Figure 25, it is unexpected that they would share more common OTUs with the algae than the 14-day old larvae. This may therefore provide evidence of alternative sources to the larval microbiome other than the algal feed, such as water or gametes from broodstock oysters.

After excluding the highly abundant Rhodobacteraceae OTU (Figure 26; Figure 29), some of the most abundant OTUs shown within Figure 27 (*Tisochrysis* lutea treated samples) were identified as belonging to the *Alcanivorax* and *Tabrizicola* genera. Bacteria of the *Alcanivorax* genera are alkane-degrading marine bacteria, most recently described in oyster cultivation as biomarkers for polyethylene (Xiao et al, 2023). The *Tabrizicola* genus (belonging to the *Rhodobacteraceae* family), although not previously described in an oyster hatchery environment, has been reported as one of the top five bacterial taxa found within the glands of wild freshwater mussels (Millar et al, 2022). Both *Alcanivorax* and *Tabrizicola* were the more abundant in larval samples than they were in *Tisochrysis lutea* (Figure 27). This suggests one of two hypothesise; either that *Tisochrysis lutea* is a source of these OTUs within the larval microbiome and that they proliferate within the larvae which fed on this culture, or that they were acquired independently from the maternal egg (Unzueta-Martínez et al, 2022) and also happened to be present within the algal culture.

Figure 29 highlights OTUs of the *Pseudophaeobacter* and *Pseudomonas* genera as highly abundant within 11-day old larvae treated with *Tetraselmis suecica*. *Pseudophaeobacter* have previously been isolated from the marine sediment (Guan et al, 2022) and reported as one of the 30 most abundant microbial taxa the microalga *Emiliania huxleyi* (Dittman et al, 2020). This *Pseudophaeobacter* was more abundant in 11-day old larvae than 14-day old larvae, suggesting that again an alternative source of the larval microbiome than *Tetraselmis suecica*

and/or more proficient proliferation in the larvae. The presence of the *Pseudomonas* bacteria has been previously described within healthy adult Pacific oysters, increasing in abundance within dead or dying animals (Colwell and Sparks, 1967). Whilst larvae cultured on different diets had different microbiomes, the diet itself does not appear to be the mechanism behind this finding.

* * *

Conclusion and future recommendations:

The microbial variation observed between larvae from different dietary groups could not be conclusively attributed to the diet itself. The data presented here aims to combine culture dependent and independent methods in order to compare the microbiomes of larvae reared under different conditions. Dietary and temperature-based treatment groups were chosen after hypothesising that these conditions are likely to affect the microbial communities present in larvae and therefore may influence larval health and hatchery production.

As was an expected result, larvae reared in the higher water temperature of 25°C displayed an increased growth rate. However, a lack of 16S amplification meant that bacterial taxa between treatment groups could not be compared, and the stability of the microbial community could not be reported upon. Recommendations for improved experimental design have therefore been suggested, mainly incorporating the use of increased larval culture volumes.

Whilst the algal cultures analysed here were purchased as axenic, bacteria were identified over time – as was reflected in both 16S and CFU data. With shellfish hatcheries operating at much larger scales and in less sterile environments, it is therefore likely that bacteria may similarly be introduced into algal cultures as they are upscaled over time in commercial environments. Microbial taxa were found to be shared between algal samples and larvae of

both dietary treatment groups, suggesting that algal cultures are a likely source of microbial contamination to larval cultures.

In line with previous anecdotal information gathered from shellfish practitioners, larvae fed *Tetraselmis suecica* in addition to *Tisochrysis lutea* showed an increased growth rate. NMDS analysis then concluded the bacterial profiles between larvae given these two different diets to be statistically significant. These differences are concluded not to be driven by species richness but by the abundance of certain taxa, mainly Actinobacteria, Bdellovibrionia and Bacilli. Despite these microbial differences, it is also possible that the addition of *Tetraselmis suecica* altered additional parameters such as water chemistry - which may have also influenced larval growth. Future research investigating the effects that these algal species alone may have on the water chemistry would therefore be highly beneficial.

In agreement with previous studies, the data presented here also shows that the bacteria of larval culture water are not proportionally transferred to larval microbiomes (Arfken et al, 2021; Unzueta-Martínez et al, 2022). The OTUs present in algal samples are also concluded to have influence over the larval microbiome, with the majority of OTUs detected in larvae also being found within corresponding algal samples. Together, this information aids in understanding the source of the larval microbiome, which is vital in order to limit microbial contamination within hatcheries and develop microbial manipulation strategies.

References:

Allen, S. K., Gaffney, P. M., & Ewart, J. W. (1993). Northeastern Regional Aquaculture Center Genetic Improvement of the Eastern Oyster for Growth and Disease Resistance in the Northeast. Northeastern Regional Aquaculture Center, University of Massachusetts, Dartmouth.

Arfken, A., Song, B., Allen, S. K., & Carnegie, R. B. (2021). Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. *Aquaculture*, *531*. https://doi.org/10.1016/j.aquaculture.2020.735955

Artimová, R., Hleba, L., Javoreková, S., Maková, J., Medová, J., & Medo, J. (2022). Chloroplast Excluding Primers For Metagenomic Analysis Of Bacteria In Plant Tissues. *Journal of microbiology, biotechnology and food sciences*, 12(3), e9650-e9650.

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*, 464, 434–444. https://doi.org/10.1016/j.aquaculture.2016.07.020

Barbosa Solomieu, V., Renault, T., & Travers, M. A. (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. *Journal of Invertebrate Pathology*, 131, 2–10. https://doi.org/10.1016/j.jip.2015.07.011

Benemann, J.R. (1992). Microalgae aquaculture feeds. *J. Appl. Phycol*. 4, 233–245.

Bousfield, I.J., Smith, G.L., Dando, T.R., Hobbs, G. (1983). Numerical analysis of total fatty acid profiles in the identification of coryneform, nocardioform and some other bacteria. *J. Gen. Microbiol*. 129, 375–394

Borzykh, O. G., & Zvereva, L. V. (2012). Mycobiota of the giant oyster *Crassostrea gigas* (Thunberg, 1787) (Bivalvia) from the Peter the Great Bay of the Sea of Japan. *Microbiology*, *81*(1), 109–111. https://doi.org/10.1134/S0026261712010031

Botta, R., Asche, F., Borsum, J. S., & Camp, E. V. (2020). A review of global oyster aquaculture production and consumption. *Marine Policy*, *117*. https://doi.org/10.1016/j.marpol.2020.103952

Boyd, C. E., McNevin, A. A., & Davis, R. P. (2022). The contribution of fisheries and aquaculture to the global protein supply. *Food Security*, *14*(3), 805–827. https://doi.org/10.1007/s12571-021-01246-9

Colwell, R. R., & Sparks, A. K. (1967). Properties of *Pseudomonas enalia*, a Marine Bacterium Pathogenic for the Invertebrate *Crassostrea gigas (*Thunberg*). Applied Microbiology,* 15(5), 980-986. https://journals.asm.org/journal/am

da Costa, F., González-Araya, R., & Robert, R. (2023). Using combinations of microalgae to condition European flat oyster (*Ostrea edulis*) broodstock and feed the larvae: Effects on reproduction, larval production and development. *Aquaculture*, *568*. https://doi.org/10.1016/j.aquaculture.2023.739302

Dittmann, K. K., Rasmussen, B. B., Melchiorsen, J., Sonnenschein, E. C., Gram, L., & Bentzon-Tilia, M. (2020). Changes in the Microbiome of Mariculture Feed Organisms after Treatment with a Potentially Probiotic Strain of *Phaeobacter inhibens*. *Applied and environmental microbiology*, 86(14), e00499-20. https://journals.asm.org/journal/aem

Dugeny, E., de Lorgeril, J., Petton, B., Toulza, E., Gueguen, Y. and Pernet, F. (2022). Seaweeds influence oyster microbiota and disease susceptibility. *Journal of Animal Ecology*, 91(4), pp.805-818.

Elston, R. A., Moore, J., & Abbott, C. L. (2012). Denman Island disease (causative agent *Mikrocytos mackini*) in a new host, Kumamoto oysters *Crassostrea sikamea*. *Diseases of Aquatic Organisms*, *102*(1), 65–71. https://doi.org/10.3354/dao02519

Enríquez-Espinoza, T. L., Grijalva-Chon, J. M., Castro-Longoria, R., & Ramos-Paredes, J. (2010). *Perkinsus marinus* in *Crassostrea gigas* in the Gulf of California. *Diseases of Aquatic Organisms*, *89*(3), 269–273. https://doi.org/10.3354/dao02199

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

Green, T. J., Siboni, N., King, W. L., Labbate, M., Seymour, J. R., & Raftos, D. (2019). Simulated Marine Heat Wave Alters Abundance and Structure of *Vibrio* Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event. *Microbial Ecology*, *77*(3), 736–747. https://doi.org/10.1007/s00248-018-1242-9

Guan, Y., Jiang, Y., Kim, Y. M., Yu, S. Y., Choi, S. H., Choe, H., Li, Z., & Lee, M. K. (2022). *Pseudophaeobacter flagellatus* sp. nov., isolated from coastal water. *International Journal of Systematic and Evolutionary Microbiology*, *72*(5). https://doi.org/10.1099/ijsem.0.005399

Hershberger, W. K., Perdue, J. A., & Beattie, J. H. (1984). Genetic selection and systematic breeding in Pacific oyster culture. *Aquaculture*, 39(1-4), 237-245.

Humphreys, J., Herbert, R. J. H., Roberts, C., & Fletcher, S. (2014). A reappraisal of the history and economics of the Pacific oyster in Britain. *Aquaculture*, *428–429*, 117–124. https://doi.org/10.1016/j.aquaculture.2014.02.034

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2021). Microbiome diversity and dysbiosis in aquaculture. In *Reviews in Aquaculture*,13(2), 1077–1096. John Wiley and Sons Inc. https://doi.org/10.1111/raq.12513

Kang, C. H., Gu, T., & So, J. S. (2018). Possible probiotic lactic acid bacteria isolated from oysters (*Crassostrea gigas*). *Probiotics and Antimicrobial Proteins*, *10*(4), 728–739. https://doi.org/10.1007/s12602-017-9315-5

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS ONE*, *7*(12). https://doi.org/10.1371/journal.pone.0051475

King, W. L., Jenkins, C., Seymour, J. R., & Labbate, M. (2019). Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment. In *Marine Environmental Research*, 43, 124–140. Elsevier Ltd. https://doi.org/10.1016/j.marenvres.2018.11.007

Leal, E., de Beyer, L., O'Connor, W., Dove, M., Ralph, P. J., & Pernice, M. (2021). Production optimisation of *Tisochrysis lutea* as a live feed for juvenile Sydney rock oysters, *Saccostrea glomerata*, using large-scale photobioreactors. *Aquaculture*, *533*. https://doi.org/10.1016/j.aquaculture.2020.736077

Lokmer, A., & Mathias Wegner, K. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME Journal*, *9*(3), 670–682. https://doi.org/10.1038/ismej.2014.160

Martínez-García, M. F., Ruesink, J. L., Grijalva-Chon, J. M., Lodeiros, C., Arreola-Lizárraga, J. A., de la Re-Vega, E., Varela-Romero, A., & Chávez-Villalba, J. (2022). Socioecological factors related to aquaculture introductions and production of Pacific oysters (*Crassostrea gigas*) worldwide. In *Reviews in Aquaculture*, 4(2), 613–629. John Wiley and Sons Inc. https://doi.org/10.1111/raq.12615

Meng, L., Xu, C., & Li, Q. (2022). Effects of Temperature, Salinity and Stocking Density on Larval Survival and Growth of Reciprocal Crosses Between Two Strains of Pacific Oysters, *Crassostrea gigas*. *Journal of Ocean University of China*, *21*(4), 1017–1026. https://doi.org/10.1007/s11802-022-4893-6

Millar, E. N., Kidd, K. A., Surette, M. G., Bennett, C. J., Salerno, J., & Gillis, P. L. (2022). Effects of municipal wastewater effluents on the digestive gland microbiome of wild freshwater mussels (*Lasmigona costata*). *Ecotoxicology and Environmental Safety*, 241. https://doi.org/10.1016/j.ecoenv.2022.113774

Offret, C., Paulino, S., Gauthier, O., Chateau, K., Bidault, A., Corporeau, C., Miner, P., Petton, B., Pernet, F., Fabioux, C., Paillard, C., & Le Blay, G. (2020). The marine intertidal zone shapes oyster and clam digestive bacterial microbiota. *FEMS Microbiology Ecology*, *96*(8). https://doi.org/10.1093/femsec/fiaa078

Pernet, F., Lupo, C., Bacher, C., & Whittington, R. J. (2016). Infectious diseases in oyster aquaculture require a new integrated approach. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1689). https://doi.org/10.1098/rstb.2015.0213

Petton, B., Destoumieux-Garzón, D., Pernet, F., Toulza, E., de Lorgeril, J., Degremont, L., & Mitta, G. (2021). The Pacific Oyster Mortality Syndrome, a Polymicrobial and Multifactorial Disease: State of Knowledge and Future Directions. In *Frontiers in Immunology*, 12. Frontiers Media S.A. https://doi.org/10.3389/fimmu.2021.630343

Pohlner, M., Dlugosch, L., Wemheuer, B., Mills, H., Engelen, B., & Reese, B. K. (2019). The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00659

Richards, G. P., Watson, M. A., Needleman, D. S., Church, K. M., & Häse, C. C. (2015). Mortalities of Eastern And Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Applied and Environmental Microbiology*, *81*(1), 292–297. https://doi.org/10.1128/AEM.02930-14

Rico-Villa, B., Le Coz, J. R., Mingant, C., & Robert, R. (2006). Influence of phytoplankton diet mixtures on microalgae consumption, larval development and settlement of the Pacific oyster *Crassostrea gigas* (Thunberg). *Aquaculture*, *256*(1–4), 377–388. https://doi.org/10.1016/j.aquaculture.2006.02.015

Ringø, E. (2020). Probiotics in shellfish aquaculture. In *Aquaculture and Fisheries*, 5(1), 1–27. KeAi Communications Co. https://doi.org/10.1016/j.aaf.2019.12.001

Robert, R., Parisi, G., Rodolfi, L., Poli, B. M., & Tredici, M. R. (2001). Use of fresh and preserved Tetraselmis suecica for feeding *Crassostrea gigas* larvae. *Aquaculture*, 192(2-4), 333-346.

Romalde, J. L., Diéguez, A. L., Lasa, A., & Balboa, S. (2014). New *Vibrio* species associated to molluscan microbiota: A review. In *Frontiers in Microbiology*, 4. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2013.00413

Salgueiro, V., Reis, L., Ferreira, E., Botelho, M. J., Manageiro, V., & Caniça, M. (2021). Assessing the bacterial community composition of bivalve mollusks collected in aquaculture farms and respective susceptibility to antibiotics. *Antibiotics*, *10*(9). https://doi.org/10.3390/antibiotics10091135

Schmitt, P., Rosa, R. D., Duperthuy, M., de Lorgeril, J., Bachère, E., & Destoumieux-Garzón, D. (2012). The antimicrobial defense of the Pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. In *Frontiers in Microbiology*, 3. Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2012.00160

Siboni, N., King, W. L., Williams, N. L., Scanes, E., Giardina, M., Green, T. J., ... & Seymour, J. R. (2024). Increased abundance of potentially pathogenic *Vibrio* and a marine heatwave co-occur with a Pacific oyster summer mortality event. *Aquaculture*, 583, 740618.

Stevick, R. J., Sohn, S., Modak, T. H., Nelson, D. R., Rowley, D. C., Tammi, K., Smolowitz, R., Lundgren, K. M., Post, A. F., & Gómez-Chiarri, M. (2019). Bacterial community dynamics in an oyster hatchery in response to probiotic treatment. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.01060

Sun, R., Chen, J., Pan, C., Sun, Y., Mai, B., & Li, Q. X. (2020). Antibiotics and food safety in aquaculture. *Journal of Agricultural and Food Chemistry*, 68(43), 11908–11919. https://doi.org/10.1021/acs.jafc.0c03996

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, *4*(1). https://doi.org/10.1186/s42523-022-00186-9

Vignier, J., Laroche, O., Rolton, A., Wadsworth, P., Kumanan, K., Trochel, B., Pochon, X., & King, N. (2021). Dietary Exposure of Pacific Oyster (*Crassostrea gigas*) Larvae to Compromised Microalgae Results in Impaired Fitness and Microbiome Shift. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.706214

Vogeler, S., Bean, T. P., Lyons, B. P., & Galloway, T. S. (2016). Dynamics of nuclear receptor gene expression during Pacific oyster development. *BMC Developmental Biology*, 16(1), 1–13. https://doi.org/10.1186/s12861-016-0129-6

Xiao, S., Zhang, Y., Wu, Y., Li, J., Dai, W., Pang, K., Liu, Y., & Wu, R. (2023). Bacterial community succession and the enrichment of antibiotic resistance genes on microplastics in an oyster farm. *Marine Pollution Bulletin*, *194*. https://doi.org/10.1016/j.marpolbul.2023.115402

Yeh, H., Skubel, S. A., Patel, H., Cai Shi, D., Bushek, D., & Chikindas, M. L. (2020). From Farm to Fingers: an Exploration of Probiotics for Oysters, from Production to Human Consumption. In *Probiotics and Antimicrobial Proteins*, 12, (2), 351–364. Springer. https://doi.org/10.1007/s12602-019-09629-3

Chapter five: Exploring the Rhodobacteraceae family for inhibitory effects against *Vibrio* infection in *Crassostrea gigas*.



Abstract:

Certain bacterial species of the *Vibrio* genus are responsible for mass mortality disease events in oyster hatcheries. Although under strict regulation, antibiotic treatments can be used to limit these disease events, thereby increasing hatchery production. However, the overuse and misuse of antibiotics leads to the development of antibiotic resistant bacterial species and is therefore not a sustainable method of bacterial treatment. This has created the need for alternative methods of bacterial disease prevention such as probiotic treatments. This chapter assesses candidate Rhodobacteraceae strains for their probiotic potential via growth inhibition of pathogenic *Vibrio aestuarianus* and *Vibrio splendidus*. These Rhodobacteraceae candidate strains were first tested *in vitro* using an agar assay before top-performing candidates were used *in vivo* during *Vibrio* bath challenge infection with adult *Crassostrea gigas* (*C. gigas*) oysters. Two candidate Rhodobacteraceae strains improved survival rate from 0% to 50% and 60% following *V. aestuarianus* infection. These promising preliminary data are discussed with a focus on recommendations for the future experimental work needed to replicate these analyses and apply such treatments to large-scale trials in a hatchery environment.

Introduction:

Pathogenic Vibrio species are a major limitation to oyster aquaculture:

Disease events are a major limiting factor to efficient production of many aquaculture species (Murray and Peeler, 2005; Stentiford et al, 2012; Stentiford et al, 2017). They also play a key role in the limitation of oyster production (Pernet et al, 2016), with large scale outbreaks causing losses of entire seedstock batches within hatcheries (Prado et al, 2010). These disease events often involve complex interactions between multiple pathogens, the host, and its environment. Bacterial pathogens are significant contributors to disease outbreaks, with the *Vibrio* genera being of particular importance to *Crassostrea gigas* (the Pacific oyster) (Green et al, 2016; Lupo et al, 2019; Richards et al, 2015). Although species of this genera have been reported within the microbiome circulatory system of healthy oysters (King et al, 2019), others are pathogenic at the larval (Richards et al, 2015; Ushijima et al, 2018), spat (Lacoste et al, 2001) and adult (Coyle et al 2023) growth stages. Bacterial diseases occur for various reasons, all of which result in the oyster microbiome being in a state of 'dysbiosis' - a term used to describe a shift in the hosts microbiome which no longer expresses beneficial synergy (Diner et al, 2023). This may occur via infiltration of the oyster by pathogenic *Vibrio* (sometimes encouraged by environmental stressors), which breakdown host immune cells and tissues. Alternatively, inhibition of the host immune system by viral pathogens allows for the colonisation of *Vibrio* bacteraemia which ultimately proves fatal to the oyster (de Logeril et al, 2018).

Two pathogenic *Vibrio* species of significance to *C. gigas* are *Vibrio splendidus* (Lacoste et al, 2001; Sugumar et al, 1998; Vezzulli et al 2015) *and Vibrio aestuarianus* (Dubert et al, 2017; Travers et al, 2017; Vezzulli et al, 2015). *Vibrio splendidus* for example has been reported as the bacterial pathogen responsible for summer mortality events of *C. gigas* in the Bay of Morlaix for over ten years (Lacoste et al, 2001). Similarly, *Vibrio aestuarianus* is known to be the cause of disease outbreaks in France since 2001, and has since also been isolated in Scotland and Ireland following mortality events (Travers et al, 2017). In the hatchery environment, these species are also a significant limiting factor of larval production, causing huge economic losses to the industry (Dubert et al, 2016; Gradoville et al, 2018). Improving our understanding of the *Vibrio* genera and exploring new ways to limit *Vibrio* disease events

is therefore an important area of research with important applications for the improvement of oyster aquaculture. This chapter aims to address this knowledge gap by investigating possible methods of limiting *Vibrio* disease outbreaks for the benefit of both wild and farmed populations.

The use of antibiotics in aquaculture:

Antibiotics are commonly used to control bacterial disease and are among the most applied chemicals in aquaculture (Lulijwa et al, 2020). Common antibiotic classes include phenicol, tetracycline and sulfonamide which allow for disease prevention and growth stimulation in fish and shrimp farms (Luu et al, 2021). Although they can immediately increase productivity in intensive aquaculture systems, their usage can also result in serious health and environmental safety issues which threaten the sustainability of the industry. Most urgently, excessive use can result in selection for antibiotic resistant bacteria and the emergence of multi-drug resistant strains (Lulijwa et al, 2020). In comparison to finfish species such as salmon (Burridge et al, 2010; Buschmann et al, 2012; Miranda et al, 2018), scientific literature contains very limited information regarding the extent or magnitude of antibiotic use within oyster hatcheries. Yeh et al 2020 reports that no antibiotics are used within oyster farms as it is a deterrent to the consumer. However, based on anecdotal evidence and discussions with industry members, it is suspected that although under strict regulation, their use in hatchery systems is both possible and common. For example, *Vibrio parahaemolyticus* resistant to ampicillin and vancomycin have been isolated from*. C. gigas*, suggesting previous exposure to these chemicals, most likely within the industry setting (Kang, 2016). The emergence of antibiotic resistant pathogens poses a significant threat oyster aquaculture, creating a need for alternative disease mitigation strategies.

The development of probiotics in aquaculture:

One alternative strategy to the use of antibiotics is the development of probiotic treatments. These are 'live or dead microbial feed supplements or water additives which improve the rearing water quality to enhance the physiological and immune responses of aquatic animals' (Hai et al, 2015). These treatments can provide a range of beneficial *in vivo* effects to the host such as antimicrobial substance production, competitive exclusion of pathogens and

immunomodulation (Yeh et al, 2020). In the farming of more advanced aquaculture species sectors, such as Atlantic salmon and Rainbow trout, probiotic treatments based on *Bacillus* and *Lactobacillus* groups of bacteria are beneficial in promoting mediation of the intestinal microbiome, regulation of immunity and benefits to mucosal health (Zhao et al, 2023; Nimalan et al, 2023).

Current studies have also begun to address the development of probiotic treatments with specific application to oyster aquaculture. Probiotic treatments of combined *Bacillus* species are beneficial to the health of *C. gigas* through improvement of water quality (Fdhila et al, 2017). This occurs via the ability of *Bacillus* species to decompose pollutants and toxic gasses such as NH_3, N_2O and H_2O_2 (Fdhila et al, 2017). The addition of *Bacillus* has also been shown as beneficial to *C. gigas* immune function by increasing haemocyte viability from 56% to 87% (Kolndadacha et al 2011). The Rhodobacteraceae family has also been characterised for its probiotic effects. Marine genera within this family include *Phaeobacter*, *Silicibacter*, *Ruegeria* and *Pseudovibrio*, strains of which produce the secondary metabolite molecule of Tropodithietic Acid (TDA) (Berger et al, 2011). Importantly, TDA production has been demonstrated to inhibit the growth of *Vibrio* species, placing Rhodobacteraceae as strong candidates for probiotic treatments. *Phaeobacter* strains isolated from mollusc hatcheries for example, are able to provide inhibitory effects on the growth of larval pathogens such as *Vibrio anguillarum* USC-72, *Vibrio neptunius* PP-145.98, and *Vibrio sp*. PP-203 (Prado et al, 2009). Other *Phaeobacter* strains have also been successfully utilised for the protection of live feed (*Artemia nauplii)* against *Vibrio anguillarum,* and for the prevention of vibriosis in cod larvae (D'Alvise et al, 2012; Grotkjær et al, 2016). These are promising developments for probiotic treatments within aquaculture and their potential transfer to bivalve rearing. Although the development and administration of such treatments will increase the overall cost of aquaculture production, they are a necessary measure in order to limit disease events in a sustainable way (Amenyogbe et al, 2020).

Chapter aims:

This chapter investigates a hypothesis which was developed as a continuation from the findings presented in chapter two: The first investigation of the environmental microbiome throughout a wild bivalve mollusc spawning event. Throughout this chapter, inverse relative abundance profiles between the *Sulfitobacter* genus (belonging to the Rhodobacteraceae family) and *Vibrio* genus were observed. Considering the existing literature on the application of marine Rhodobacteraceae and the vibriostatic effects of TDA production, the following hypotheses were investigated:

1. Candidate Rhodobacteraceae strains reduce *Vibrio splendidus* growth *in vitro*.
2. Candidate Rhodobacteracea*e* strains reduce the severity of *Vibrio splendidus* and *Vibrio aestuarianus* infection in adult *C. gigas* individuals.

Methods:

Measuring Vibrio growth inhibition using agar plate assays:

Cultures of 14 different Rhodobacteraceae strains covering genera of *Jannaschia*, *Mativia*, *Oceanibulbus* and *Sulfitobacter* (Table 1), were obtained from the culture collection at SAMS (Scottish Association of Marine Science) on Zobell Marine 10 (ZM10) agar plates. ZM10 broth media was prepared by combining 75% sterile seawater (30 ppt), 0.01% yeast extract (MilliporeSigma) and 0.05% proteose peptone (MilliporeSigma) with marine trace elements and vitamins as outlined in Green et al, 2004. Each strain was grown from a single colony in a liquid culture of ZM10 broth media held at 25˚C with shaking at 180 rpm. After 72 hours, each strain was diluted to an OD of 0.2 under aseptic conditions with additional ZM10 broth. For each of the 14 strains, 100 µL of liquid culture was spread evenly onto one half of a ZM10 agar plate and grown into a lawn at room temperature. Once a lawn of Rhodobacteraceae had been established after three to four days, liquid cultures of *Vibrio splendidus* (strain 1_C04a) were diluted to an OD of 0.2. Spots of 2 µL of *Vibrio* were spotted at regular intervals across the entire plate (Figure 1). Plates were left in the dark at room temperature for 10 days before being photographed using a V750 PRO Epson scanner.

Table 1: Strain identifications of the 14 Rhodobacteraceae strains used, their corresponding bacterial genus and origin. Symbols of '*' indicate strains which were later used in the bath challenge as suspected inhibitors of *Vibrio.* Symbols of '**' indicate strains which were used in the bath challenge as negative controls after showing no inhibitory effects against *Vibrio*.

Plate ID	Genus	Origin
855*	*Sulfitobacter*	*Gymnodinium catenatum* GCDE08
1159*	*Sulfitobacter*	*Pseudo-nitzschia* multiseries CLN-1
1254	*Sulfitobacter*	*Lingulodinium polyedrum* CCAP 1121/2
1704	*Sulfitobacter*	Oil-amended free-living bacterioplankton microcosm
1572*	*Sulfitobacter*	*Coccolithus braarudii* AC392 / RCC 1203
1492	*Sulfitobacter*	*Coccolithus braarudii* AC400
1449	*Marivita*	*Emiliania huxleyi* AC472
1985 (882)	*Marivita*	*Gymnodinium catenatum* GCDE08
1128	*Marivita*	*Gymnodinium catenatum*
1424	*Unknown*	Unknown
877	*Jannaschia*	*Gymnodinium catenatum* GCDE08
1398**	*Jannaschia*	*Emiliania huxleyi* CCAP 920/8
1153	*Oceanibulbus*	*Pseudo-nitzschia* multiseries CLN-1
1986(Pn24)	*Marivita*	*Nannochloropsis*

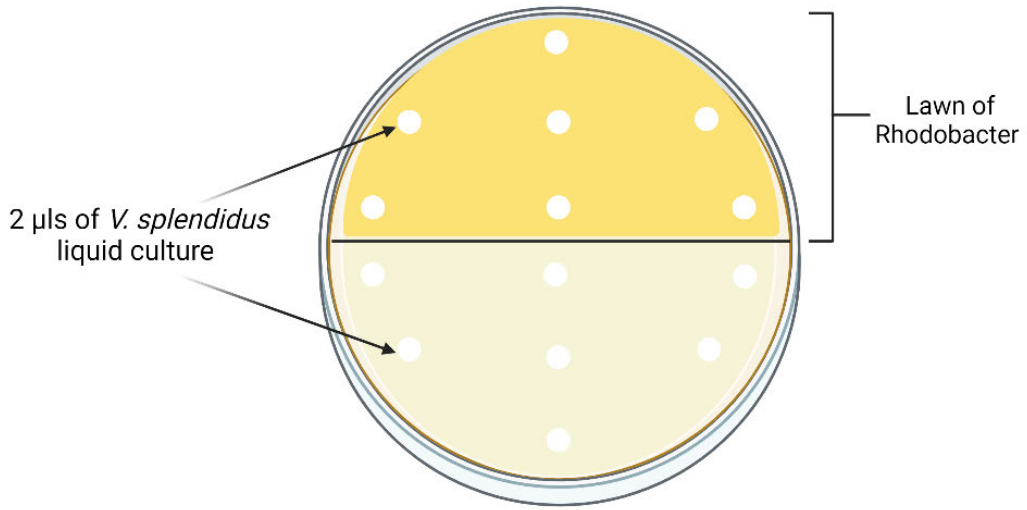


Figure 1: Design of the plate assay used to test the effect of Rhodobacteraceae on the growth of *Vibrio splendidus.* Using a ZM10 agar plate, the Rhodobacteraceae strain of interest is grown as a lawn on one half of the plate. A liquid culture of *Vibrio splendidus* is then spotted in an even distribution over the entire plate, allowing the assessment of direct and indirect growth inhibition.

Growth of Rhodobacteraceae strains for preliminary bath challenges:

Selected *Rhodobacteraceae* strains were maintained in liquid culture. This was done by selecting single colonies with a from ZM10 agar plate with a 1 µL inoculation loop and inoculating 25 mLs of ZM10 broth with 1X Marine Supplement added using strict aseptic technique. Each liquid culture was held in a 50 mL Falcon tube for 48 hours at 25°C at 180pm to provide aeration.

Growth of Vibrio strains for preliminary bath challenges:

The *Vibrio splendidus* strain 1_C04a and one strain of *Vibrio aestuarianus* (*V. a.*) were prepared in cultures of liquid marine broth (Marine 2216, Merck KGaA, Darmstadt, Germany). This was done by selection single colonies from cultures on marine agar plates with a 1 µL inoculation loop in order to inoculate the 25mLs of the marine broth. Each liquid culture was held in a 50 mL Falcon tube for 48 hours at 20°C with shaking at 180rpm.

Bath challenges:

After observing growth patterns on the agar plate assays, four Rhodobacteraceae strains (885, 1159, 1572 and 1398) were selected for use in *C. gigas* bath challenge infection trials (Table 1). Within these, strain 1398 functioned as a negative control after showing no inhibitory effects against the growth of *V. splendidus*. In bath challenge experiments, each strain of *Rhodobacteraceae* was tested for inhibitory effects again both *V. s*. and *V. a.* in triplicate. Control treatment groups (Figure 2) were included where oysters were given the following treatments:

1. *V. splendidus* only
2. *V. aestuarianus* only
3. *Rhodobacteraceae* strain 885 only
4. *Rhodobacteraceae* strain 1159 only
5. *Rhodobacteraceae* strain 1572 only
6. *Rhodobacteraceae* strain 1398 only
7. No bacterial treatment

C. gigas individuals were obtained from Lindisfarne Oysters (UK) with anterior to posterior shell height ranging from 6.5 – 8.2 cm. All oysters were acclimatised overnight in aerated artificial seawater of 30 ppt at 25˚C. Following this acclimatisation, oysters were placed in individual transparent plastic cups which contained 150 mL of aerated sterile artificial seawater of 30 ppt and incubated at 25˚C. Liquid cultures of each Rhodobacteraceae strain were centrifuged at 6,000 x g for 10 minutes in order to pellet the bacterial cells. The growth media was then removed using a pipette and the bacterial pellet was resuspended in sterile artificial seawater. Rhodobacteraceae cultures were then added to the necessary beakers (Figure 2) to a concentration of $1x10^6$ CFU/mL. Liquid cultures of *V. a.* and *V. s.* were prepared in the same way and added to the necessary beakers 24 hours later at $1x10^6$ CFU/mL. Oysters were held in an incubator maintained at a temperature of 20°C. Oysters were then checked daily for the next 7 days oyster mortalities recorded. The water within each cup was replaced with new sterile, aerated sea water (30 ppt) on alternating days. These water changes ensured that water quality was kept consistent throughout the experiment. Photographs taken throughout this preliminary challenge can be seen in Figure 3 which includes examples of oyster mortalities.

Data analysis:

Results of the bath challenge experiment were processed using GraphPad Prism (v 10.3.1) to create survival curves over time of control vs treatment groups. To test for statistical significance between these groups, Log-rank (Mantel-Cox) tests were carried out, also using the GraphPad Prism (v 10.3.1) software.

Biosecurity measures:

All excess bacterial cultures, waste water and individual oysters were disposed of in accordance with the Roslin institutes biosafety protocols. Liquid bacterial cultures were inactivated in a 5% Virkon solution for 24 hours before being disposed of. Bacterial cultures on agar plates and individual oysters were incinerated via the Roslin Institutes clinical waste disposal route. All wastewater was held for a minimum of 6 hours and treated 1:100 with a dilution of 13% Sodium hypochlorite (Fisher Scientific) resulting in >1000 ppm available chlorine, before being disposed of in normal freshwater sewage. Sewage was treated at Gorebridge secondary treatment WWTW, and effluent eventually discharged into the Gorewater river (Lat 55.840, Long -3.060).

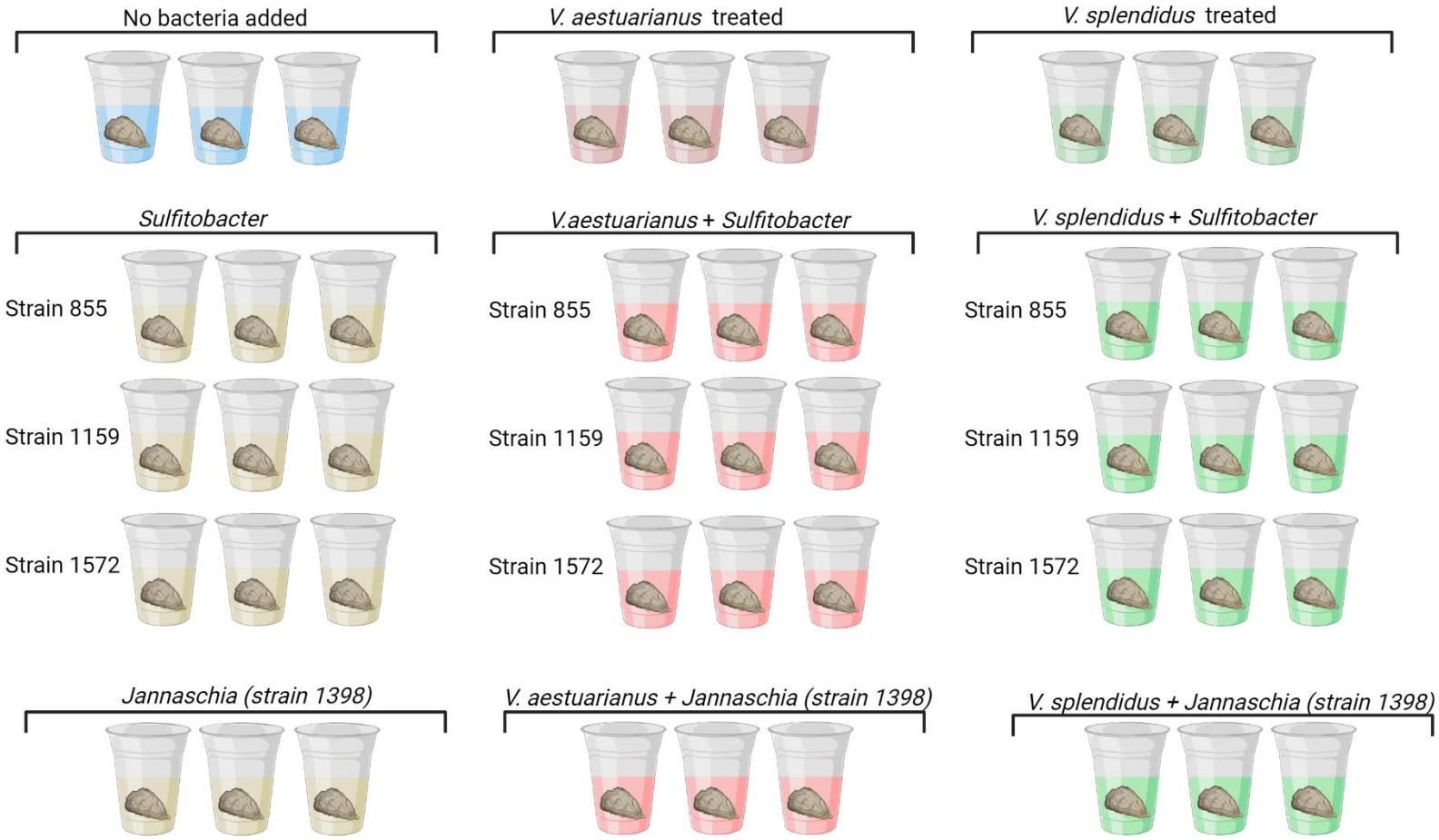


Figure 2: Bath challenge set up of *adult Crassostrea gigas* individuals challenged with *Vibrio splendidus or Vibrio aestuarianus* alongside four different strains of Rhodobacteraceae. The Rhodobacteraceae strain given to each individual are shown by the numbers 855, 1159, 1572 (belonging to the *Sulfitobacter* genus) and 1398 (belonging to the *Jannaschia genus*).

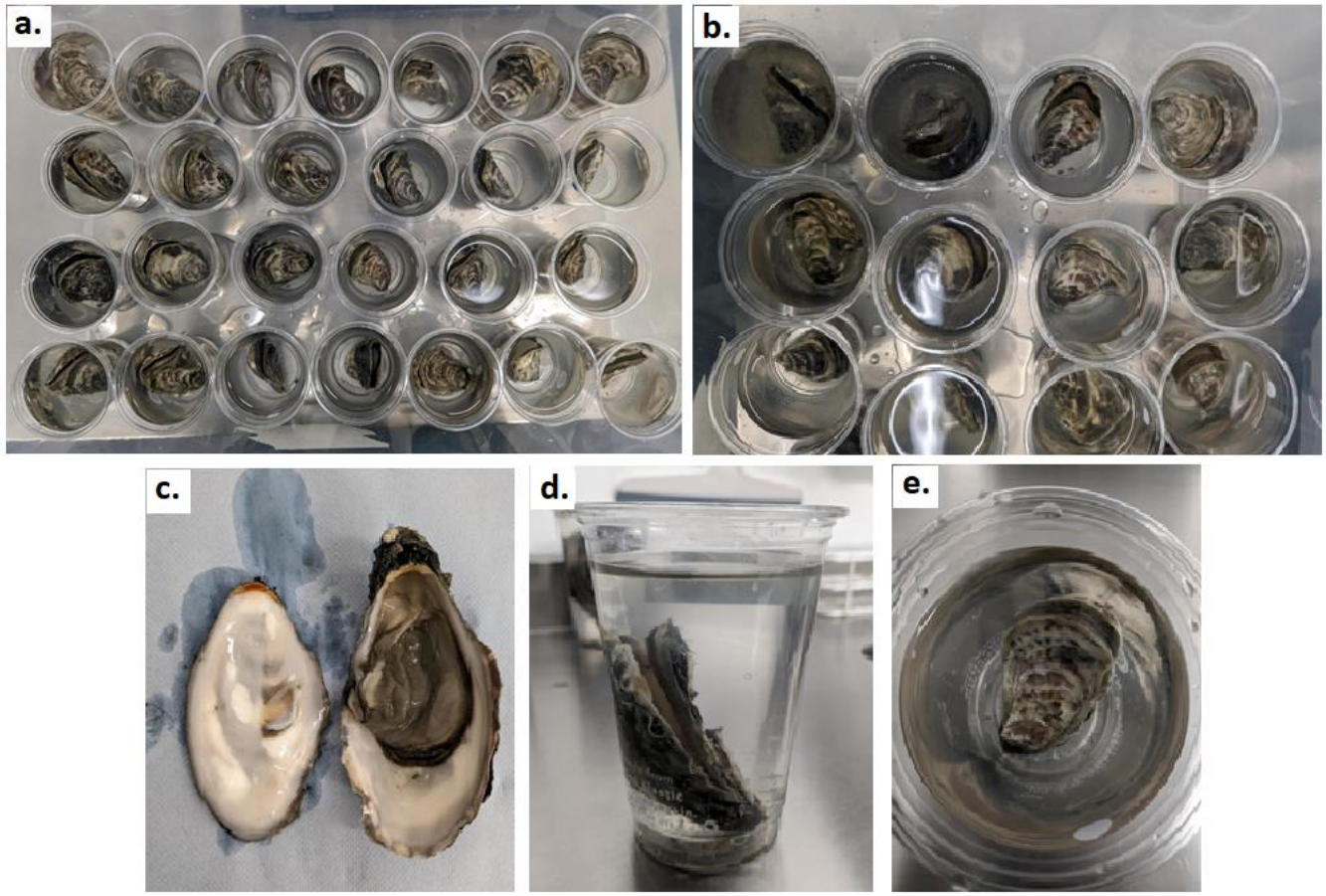


Figure 3: Photographs taken during probiotic bath challenge experiments with Pacific oysters. **(a)** Layout of individual oysters held in cups. **(b)** Showing discolouration of water sometimes observed in cups where oyster mortalities were recorded. **(c)** Opened shell following an oyster mortality. **(d)** side profile of an oyster mortality held in a cup. **(e)** Top view of an oyster mortality held in a cup.

Results:

Measuring Vibrio growth inhibition using agar plate assays:

Rhodobacteraceae strains showed a variety of visible effects against the growth of *V. s.* (Figure 4). Five strains (1986, 1449, 1128, 1398 and 1985) showed no visible effect, where spots of *V. s.* had a similar appearance regardless of the proximity to the lawn of Rhodobacteraceae. The remaining 9 strains however, showed moderate (1424, 1492, 1254, 1704, 877 and 1153), or a strong (855, 1159 and 1572) effect, where spots of *V*. s. appeared less dense when plated on or near to the lawn of Rhodobacteraceae. In some instances (877, 885 and 1159), a gradient effect was observed, where spots of *V*. s. appeared less dense as the proximity to the Rhodobacter lawn increased (Figure 5). As they were seen to have the strongest effect on the growth of *V. s.* strains 855, 1159 and 1572 (all belonging to the *Sulfitobacter* genus) were used in the preliminary bath challenge alongside strain 1398 (belonging to the *Jannaschia* genus) as a negative control.

Bath challenge:

Unexpectedly, a reduced survival rate over time was observed amongst oysters given no bacterial treatment. This treatment group reached a 66% survival rate at day 4, which reduced to 0% at day 8 (Figure 6). In comparison, groups treated with one of the four Rhodobacteraceae strains had either a higher survival rate at day 7 (Figures 6b and 6c), or took longer to reach a 0% survival rate (Figures 6a and 6d). Results of a Log-rank Mantle-Cox test reported no significant different between the survival rate of oysters given no bacterial treatment vs oysters given Rhodobacteraceae strain 1572 ($p = 0.0701$, Chi square = 3.282), 855 ($p = 0.7439$, Chi square = 0.1067), 1159 ($p = 0.8204$, Chi square, 0.05152) or 1398 ($p = 0.8204$, Chi square = 0.05152).

All oysters treated with *V. a.* survived until day 5, when survival rate then decreased dramatically to 0% (Figure 7). Alongside infection with *V. a.* oysters treated with Rhodobacteraceae strain 855 took an additional 2 days to reach the same 0% survival rate (Figure 7b). This comparison was not statistically significant ($p = 0.9219$, Chi square =

0.009615). Survival rate was significantly increased by treatment with strain 1398 by 33% (Figure 7a); (p = 0.0009, Chi square = 11.00), 1572 by 66% (Figure 7c); (p = 0.0068, Chi square = 7.333), and 1159 by 50% (Figure 7d); (p = 0.0082, Chi square = 7.00).

Oysters challenged with *V. s.* reached a 66.6% survival rate at day 7 (Figure 8). This survival rate was not increased by the addition of any Rhodobacteraceae strain. Comparisons between oysters treated with *V. s.* only vs those treated with *V. s.* and Rhodobacteraceae strains 1572 and 855 were not statistically significant (Figure 8c); (p = 0.5514, Chi square = 0.3548) and (Figure 8b); (p=0.3213, Chi square = 0.9838). Oysters treated with Rhodobacteraceae strain 1159 showed a significantly reduced survival rate in comparison to those treated with only *V. s.* (Figure 8d); (p = 0.0218, Chi square = 5.261). Mortality rate was only delayed, (although not significantly), in oysters also treated with the negative control strain 1398 (Figure 8a); (p = 0.5637, Chi square = 0.3333).

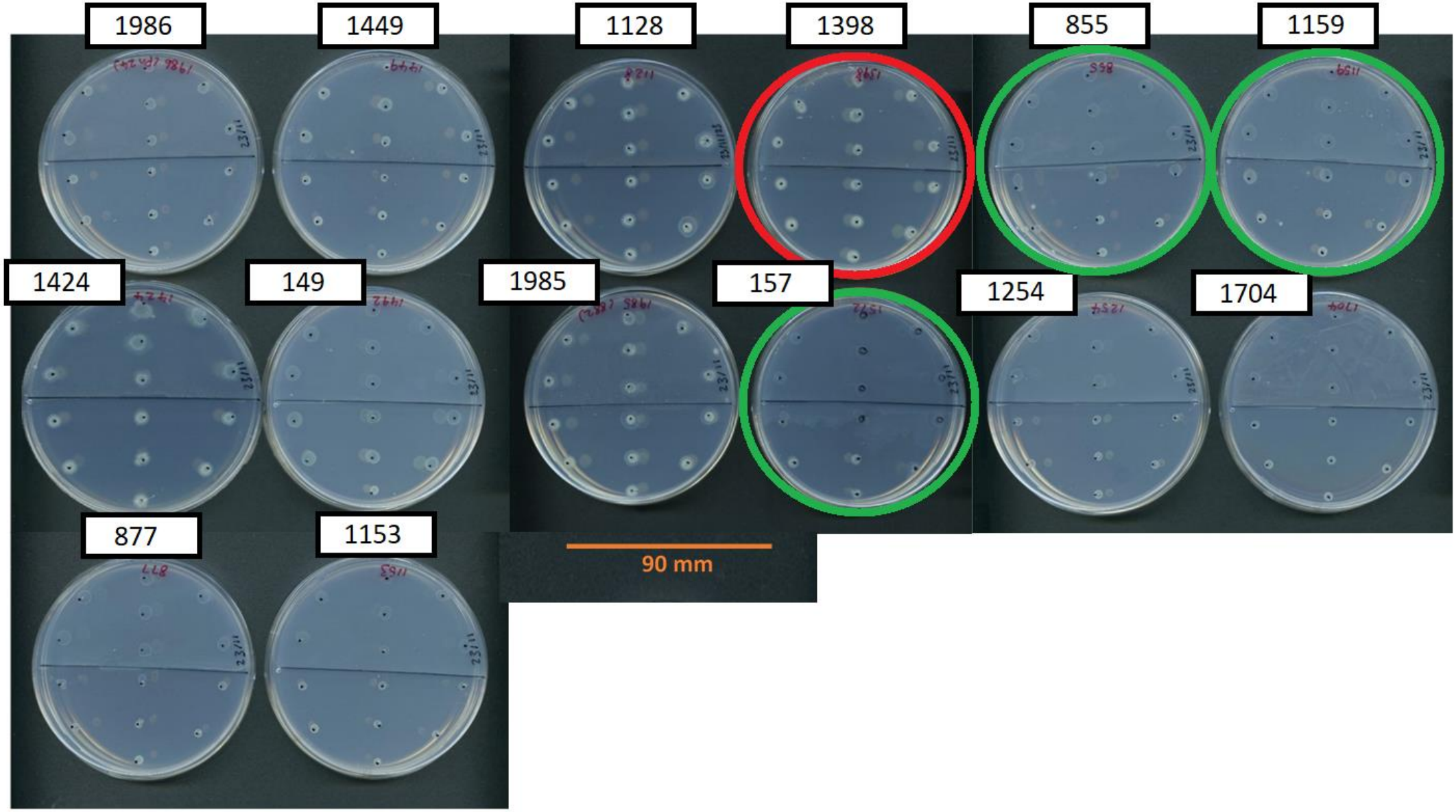

1986
1449
1128
1398
855
1159
1424
149
1985
157
1254
1704
877
1153
90 mm

Figure 4: Photographs taken of *V. s.* growth inhibition assay plates aften ten days. Each agar plate represents a different strain of Rhodobacteraceae (as indicated by the numbers shown), which was used to lawn the top half of the plate. The growth of *V. s.* can be compared on an off each Rhodobacteraceae lawn. Plates highlighted in green indicate Rhodobacteraceae strains which appeared to have an inhibitory effect on the growth of *V. s.* and which were selected for further use in preliminary bath challenges. The Rhodobacteraceae strain highlighted in red shown an example of no inhibition of *V. s.* growth and was therefore the strain selected as a negative control for the preliminary bath challenge.

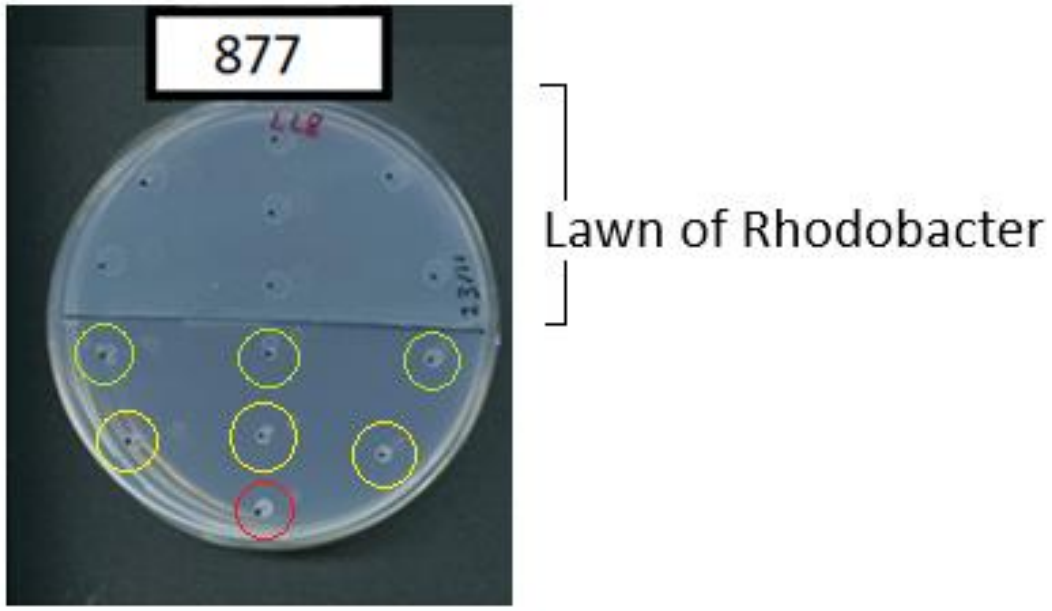


Figure 5: A close up, annotated image of the agar plate assay created with Rhodobacter strain 877. Spots of V. s growth appear most prominent furthest away from the Rhodobacter lawn (annotated with a red circle). As proximity to the Rhodobacter lawn increases, growth spots of V. s become less dense (annotated with yellow and green circles).

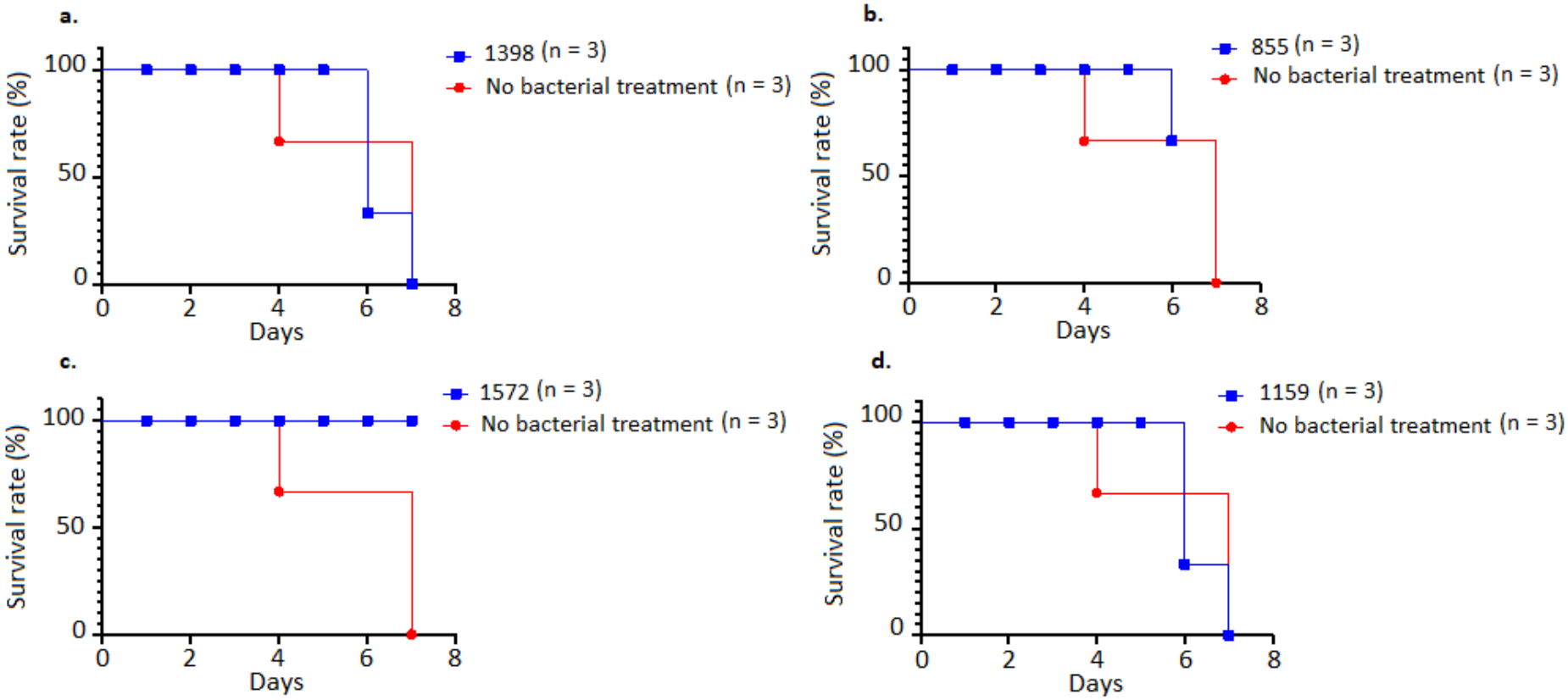


Figure 6: Mortality rates over eight days of *Crassostrea gigas* exposed to four different strains of Rhodobacteraceae **(a)** 1398 **(b)** 855 **(c)** 1572 and **(d)** 1159. Data shown in red 'no bacterial treatment', indicates control group animals where neither Rhodobacteraceae nor *Vibrio* were stains were added. Each treatment group consisted of three individual oysters.

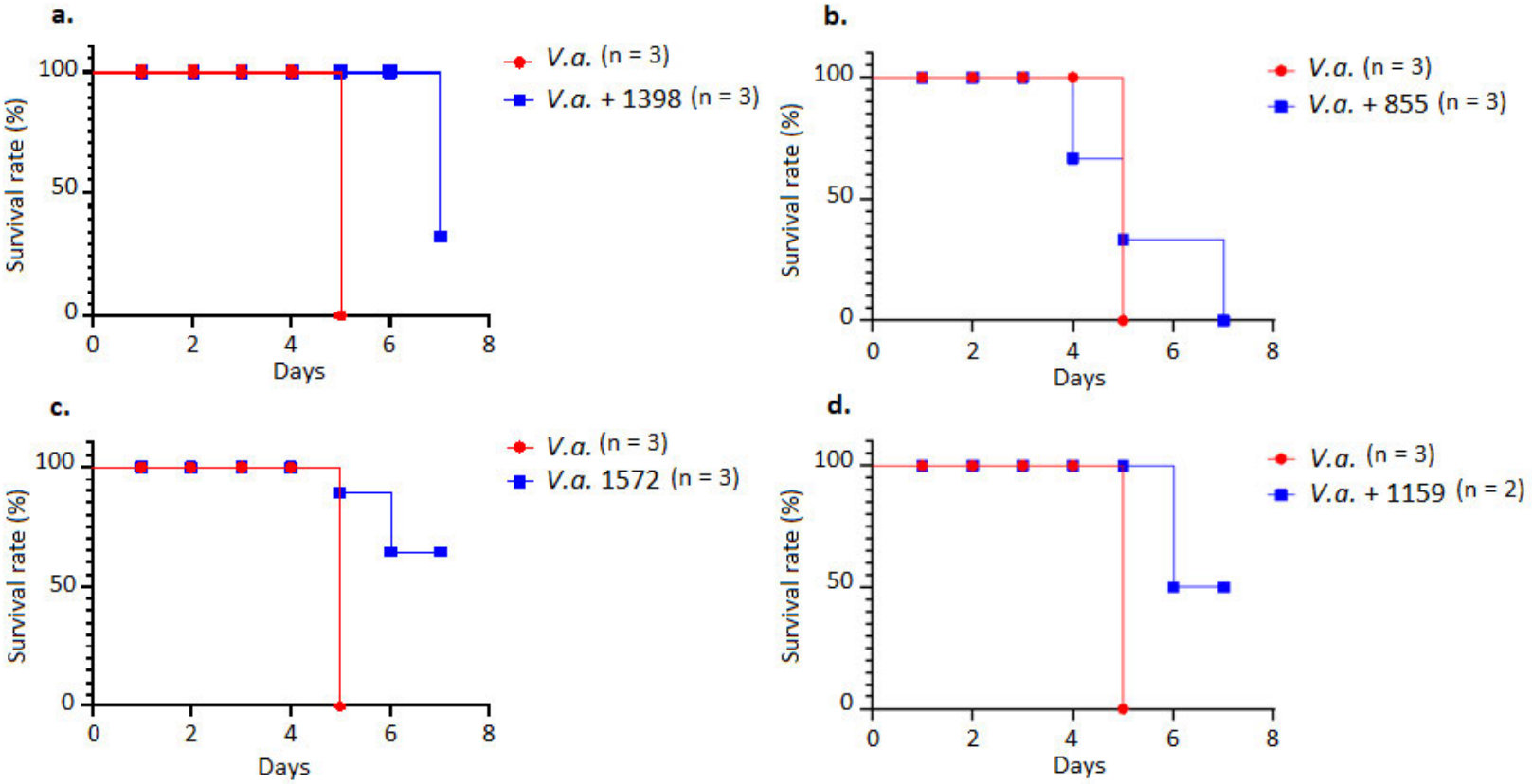


Figure 7: Mortality rates of *Crassostrea gigas* over eight days after being challenged with *V. aestuarianus (V. a.)* and one of four different strains of Rhodobacteraceae **(a)** 1398 (Negative control), **(b)** 855, **(c)** 1572, and **(d)** 1159. Each treatment group consisted of three individual oysters aside from those treated with *V. a.* and strain 1159, where sample size was reduced to two oysters due to a mortality before the addition of *V. a.* to the bath challenge.

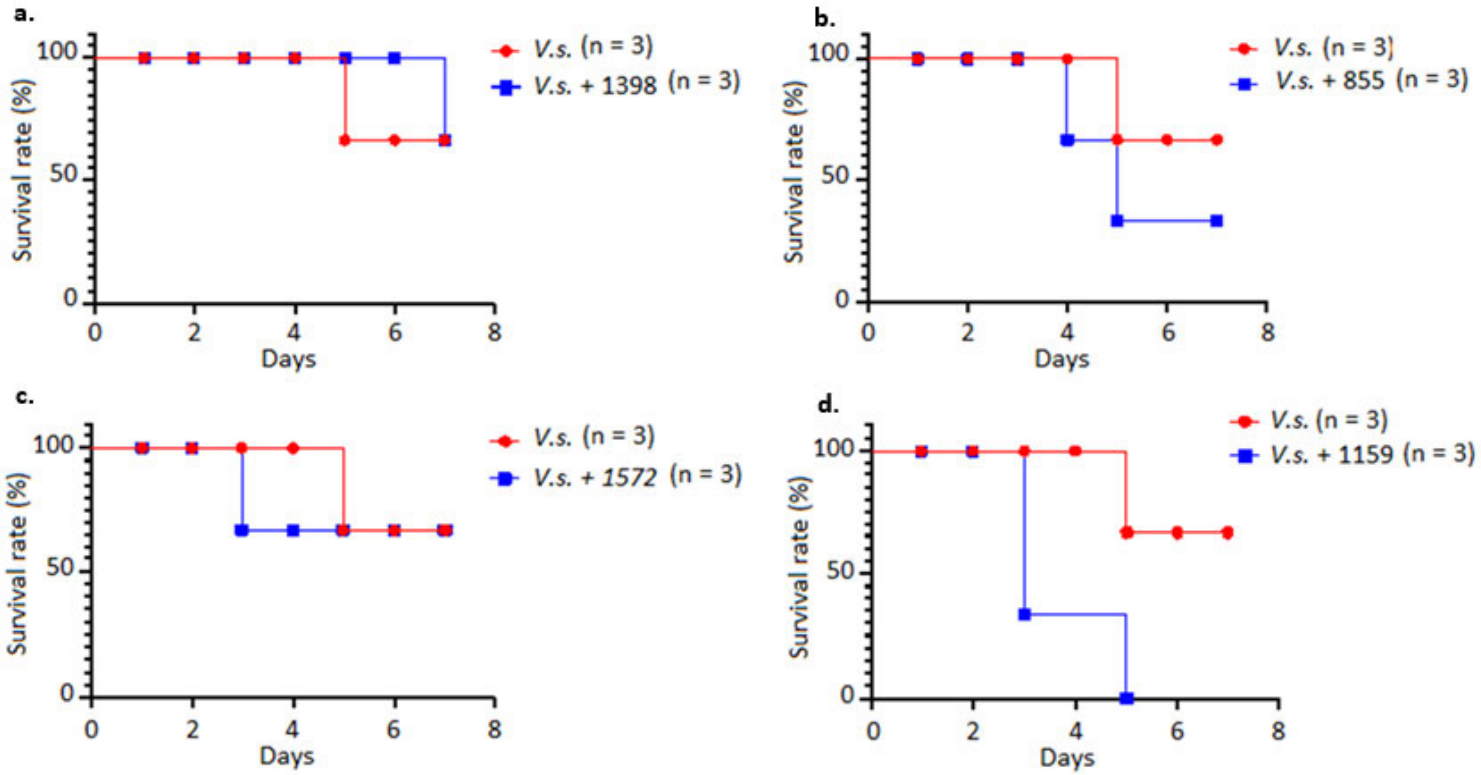


Figure 8: Mortality rates of *Crassostrea gigas* over eight days after being challenged with *V. splendidus (V. s.)* and one of four different strains of Rhodobacteraceae **(a)** 1398 (Negative control), **(b)** 855, **(c)** 1572, and **(d)** 1159. Each treatment group consisted of three individual oysters.

Discussion:

Measuring Vibrio growth inhibition using agar plate assays:

Within this chapter two independent methods were used to assess the potential of several *Rhodobacteraceae* strains to inhibit growth of pathogenic *Vibrio* species. The use of agar plate assays allows for the mass screening of many possible probiotic candidates and so are a necessary stage in probiotic selection strategies (Karim et al, 2013). In comparison to *V. s.* spotted on plain agar, *V. s.* growth appeared less dense when spotted on lawns of candidate Rhodobacteraceae strains 1424, 1492, 1254, 1704, 877, 1153, 855, 1159 and 1572 (Figure 4). This suggests competition or inhibition of *Vibrio* growth by the Rhodobacteraceae. When strains 877, 885 and 1159 were used, a more distinct, gradient like, effect can be seen, where spots of *V. s.* appear to have grown less dense with increasing proximity to the lawn of Rhodobacteraceae (Figure 4; Figure 5). This evidence suggests that these strains are able to inhibit the growth of *V. s.* through a mechanism other than direct competition of growth.

It is worth noting the results are preliminary trails using Rhodobacteraceae strains not previously described within the current literature. Therefore, further research is needed to confirm this finding. Most importantly, future work should aim to repeat this experiment with biological replication of each Rhodobacteraceae strain to confirm the repeatability and reproducibility of this assay design and findings. Future works would also benefit from expanding this assay to include the testing of inhibition against additional *Vibrio* species of concern to oyster cultivation such as *V. a.*, *Vibrio jasicida, Vibrio tubiashii* and *Vibrio rotiferianus* (Elston et al, 2008; Harrison et al, 2022; Siboni et al, 2024).

Bath challenges:

After observing results from the plate assays (Figure 4), Rhodobacteraceae strains 855, 1159 and 1572 were tested for their potential probiotic effects in a whole animal immersion challenge. Strain 1398 was included as a negative control after no effect was seen on *V. s.* growth in the agar plate assays (Figure 4). This study was a preliminary experiment and had some major limitations which should be outlined. Oysters given no bacterial treatment all

died by day 7 (Figure 6) suggesting conditions in the experimental system were not favourable. However, there were nonetheless some interesting results which should be further explored. For example, although bacterial treatments of strains 1159 and 1398 also resulted in a 0% survival rate at 7 days, the first mortalities in these groups were delayed in comparison to oysters given no bacterial treatment. Also, when oysters were treated with strains 855 and 1572, overall survival rate was increased with to 66.6% and 100% retrospectively (Figure 6). Although replication of these results is required, this suggests beneficial effects of the Rhodobacteraceae added.

Oysters challenged with *V. a.* only (Figure 7), all died on day five. This suggests that the quantities of *V. a.* administered were successful in creating an effective disease challenge. The addition of strain 855 did not increase the end survival rate of oysters, however oyster mortalities were delayed in comparison to those treated with *V. a.* only (Figure 7). Addition of strains 1398, 1572 and 1159 during *V. a.* challenge significantly increased overall survival rate to 33.3, 66.6 and 50% respectively (Figure 7). Although strain 1398 was not expected to show any effect on oyster survival based on the agar plate assay, this small increase in survival rate was still observed. Unfortunately, a mortality was observed in the *V. a.* with 1159 treatment group before the addition of *V. a.,* making this group a sample size of two. Circumstances such as this make an increase in sample size one of the most important future recommendations to further this work. Therefore, whilst the results presented are promising in suggesting that strains 1398, 1572 and 1159 may confer resistance in oysters against infection with *V. a,* the reproducibility of this data within larger sample sizes is again required.

In contrast to the dramatic mortality rate observed in oysters challenged with *V. a.* only, those given *V. s.* only resulted in a 66.6% survival rate at the end of the eight-day experiment (Figure 8). This high survival rate suggests that the *V. s.* dosage was not sufficient enough for an effective bacterial challenge. Importantly, oysters treated with strains 1398 and 1572 as well as *V. s.* also resulted in an overall survival rate of 66.6%, whilst the addition of strains 885 and 1159 resulted in lower survival rates of 33.3 and 0% respectively (Figure 8). Within these bath challenges, the uptake of bacteria from the water is reliant upon active water filtration from the oysters. It is possible that the oysters within this challenge were not sufficiently filtering water and did not take in the threshold level of *V. s*. needed required for achieving high levels of mortality. In order to conclude the effects of Rhodobacteraceae on bacterial infection, it is

first vital that mortalities recorded during bath challenge experiments are assured to be a result of infection with the pathogen of interest. A key recommendation to continue this research would therefore be to repeat this experiment with a higher concentration of *V. s.*, starting with $1x10^7$ CFU/mL. Furthermore, housing individual oysters in larger volumes of water with consistent aeration may reduce stress on the animal and increased filtration rates. Alternatively, different methods of administering the bacterial pathogen could be used such as direct injection into the adductor muscle of the oyster (Meng et al, 2015).

Applications to the oyster culture environment:

The development of a probiotic treatment requires consideration of many key factors depending on the target species. *C. gigas* is known to be affected by bacterial pathogens as larvae, juveniles and adults (Elston, 1993), and so probiotic treatments would be beneficial to any stage of the life cycle. However, probiotic treatments would be arguably ideally administered to larvae. This is since oysters are most susceptible to bacterial diseases at this stage, and larval health is imperative for the seedstock production that oyster aquaculture is so heavily reliant upon. Another recommendation to further the work presented here, would therefore be to repeat these bath challenges using oyster larvae. The success of a probiotic treatment at this life stage could improve survival rate at this crucial point in the hatchery process. Probiotic treatments could also be highly beneficial when administered to adult oysters if used during broodstock conditioning. Although this is not a method of broodstock conditioning discussed within the current literature, there is evidence to suggest that the microbiome of larvae can be influenced by the bacteria present in the maternal egg (Unzueta-Martínez et al, 2022). It is therefore possible that beneficial effects from probiotic treatments given to broodstock may also be conferred to the subsequent larvae. Regardless of the life stage at which they are administered, the development of probiotic treatments holds great potential to improve oyster production. Probiotics may confer resistance against single pathogens (such as *V.a.* and *V.s* discussed here), but could in turn also decrease the prevalence of diseases which result as a matter of co-infection with such *Vibrio* species. For example, the development of OsHV-1 in *C. gigas* is initiated by bacteriemia caused by proliferation of *Vibrio* bacteria (Chapter 1, section 3.4). Future experiments may therefore

also aim to investigate the effects of probiotic candidates on *C. gigas* individuals in a state of common and destructive co-infection (such as OsHV-1).

Conclusion:

Firstly, this chapter outlines an agar plate assay design for the initial selection of three Rhodobacteraceae probiotic candidates. This assay design provides an effective method of initial *in vitro* screening and could be replicated for the mass screening of any future probiotic candidate. Bath challenge infection experiments reported promising preliminary data that Rhodobacteraceae *Sulfitobacter* strains 1159 and 1572 may be possible probiotic candidates for the protection *C. gigas* against infection with *V. a.* Immediate future work should focus on reproducing the results presented here with treatment groups of increased sample size. If successful, this would then allow for the screening of these probiotic candidates against other key pathogens such as *Vibrio jasicida*, *Vibrio tubiashii* and *Vibrio rotiferianus*. This is preliminary data, using unique experimental design with Rhodobacteraceae strains which have not previously been investigated for their probiotic potential. Future work would therefore be expected to involve repetition of these experiments to incorporate: variations in the concentrations of *Vibrio* and Rhodobacteracea*e* bacteria administered; alternative methods of pathogenic exposure and the testing of oysters at all stages of the life cycle. The development of probiotic treatments is a vital measurement in order to sustainably prevent bacterial disease outbreaks in oyster hatcheries.

References:

Amenyogbe, E., Chen, G., Wang, Z., Huang, J. S., Huang, B., & Li, H. (2020). The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions. : a review. In *Aquaculture International*, 28 (3), 1017–1041. Springer. https://doi.org/10.1007/s10499-020-00509-0

Berger, M., Neumann, A., Schulz, S., Simon, M., & Brinkhoff, T. (2011). Tropodithietic acid production in Phaeobacter gallaeciensis is regulated by N-acyl homoserine lactone-mediated quorum sensing. *Journal of Bacteriology*, *193*(23), 6576–6585. https://doi.org/10.1128/JB.05818-11

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., & Bostick, K. (2010). Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. In *Aquaculture*, 306 (1–4), 7–23. https://doi.org/10.1016/j.aquaculture.2010.05.020

Buschmann, A. H., Tomova, A., López, A., Maldonado, M. A., Henríquez, L. A., Ivanova, L., Moy, F., Godfrey, H. P., & Cabello, F. C. (2012). Salmon aquaculture and antimicrobial resistance in the marine environment. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042724

Coyle, N. M., O'toole, C., Thomas, J. C. L., Ryder, D., Feil, E. J., Geary, M., Bean, T. P., Joseph, A. W., Waine, A., Cheslett, D., & Verner-Jeffreys, D. W. (2023). *Vibrio aestuarianus* clade A and clade B isolates are associated with Pacific oyster (*Magallana gigas*) disease outbreaks across Ireland. *Microbial Genomics*, 9(8). https://doi.org/10.1099/mgen.0.001078

D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø., & Gram, L. (2012). *Phaeobacter gallaeciensis* reduces *vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS ONE*, 7(8). https://doi.org/10.1371/journal.pone.0043996

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J. M., Haffner, P., Dégremont, L., Charrière, G. M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., … Mitta, G. (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06659-3

Diner, R. E., Zimmer-Faust, A., Cooksey, E., Allard, S., Kodera, S. M., Kunselman, E., Garodia, Y., Verhougstraete, M. P., Allen, A. E., Griffith, J., & Gilbert, J. A. (2023). Host and Water Microbiota Are Differentially Linked to Potential Human Pathogen Accumulation in Oysters. *Applied and Environmental Microbiology*, *89*(7). https://doi.org/10.1128/aem.00318-23

Dubert, J., Osorio, C. R., Prado, S., & Barja, J. L. (2016). Persistence of Antibiotic Resistant Vibrio spp. in Shellfish Hatchery Environment. *Microbial Ecology*, 72(4), 851–860. https://doi.org/10.1007/s00248-015-0705-5

Dubert, J., Barja, J. L., & Romalde, J. L. (2017). New insights into pathogenic vibrios affecting bivalves in hatcheries: Present and future prospects. In *Frontiers in Microbiology*, 8. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2017.00762

Ralph A. Elston. (1993). Infectious diseases of the Pacific oyster, *Crassostrea giga*s. *Annual Review of Fish Diseases*, 3, 259-276. ISSN 0959-8030, https://doi.org/10.1016/0959-8030(93)90038-D.

Elston, R. A., Hasegawa, H., Humphrey, K. L., Polyak, I. K., & Hase, C. C. (2008). Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: Severity, environmental drivers, geographic extent and management. *Diseases of Aquatic Organisms*, 82(2), 119–134. https://doi.org/10.3354/dao01982

Fdhila, K., Haddaji, N., Chakroun, I., Dhiaf, A., Macherki, M. E. E., Khouildi, B., Lamari, F., Chaieb, K., Abid, N., Marzougui, H., Khouadja, S., & Missaoui, H. (2017). Culture conditions improvement of *Crassostrea gigas* using a potential probiotic *Bacillus* sp strain. *Microbial Pathogenesis*, 110, 654–658. https://doi.org/10.1016/j.micpath.2017.07.017

Gradoville, M. R., Crump, B. C., Häse, C. C., & White, A. E. (2018). Environmental Controls of Oyster-Pathogenic *Vibrio spp*. in Oregon Estuaries and a Shellfish Hatchery. https://journals.asm.org/journal/aem

Green, D. H., Llewellyn, L. E., Negri, A. P., Blackburn, S. I., & Bolch, C. J. S. (2004). Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. *FEMS Microbiology Ecology*, *47*(3), 345–357. https://doi.org/10.1016/S0168-6496(03)00298-8

Green, T. J., Vergnes, A., Montagnani, C., & De Lorgeril, J. (2016). Distinct immune responses of juvenile and adult oysters (*Crassostrea gigas*) to viral and bacterial infections. *Veterinary Research*, 47(1). https://doi.org/10.1186/s13567-016-0356-7

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P., Dourala, N., Nielsen, K. F., & Gram, L. (2016). Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in Artemia cultures. *Systematic and Applied Microbiology*, *39*(3), 180–188. https://doi.org/10.1016/j.syapm.2016.01.005

Hai, N. V. (2015). The use of probiotics in aquaculture. In *Journal of Applied Microbiology*, 119(4), 917–935. https://doi.org/10.1111/jam.12886

Harrison, J., Nelson, K., Morcrette, H., Morcrette, C., Preston, J., Helmer, L., Titball, R. W., Butler, C. S., & Wagley, S. (2022). The increased prevalence of *Vibrio* species and the first reporting of *Vibrio jasicida* and *Vibrio rotiferianus* at UK shellfish sites. *Water Research*, 211. https://doi.org/10.1016/j.watres.2021.117942

Kang, C. H., Shin, Y. J., Kim, W. R., Kim, Y. G., Song, K. C., Oh, E. G., Kim, S. K., Yu, H. S., & So, J. S. (2016). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated

from oysters in Korea. *Environmental Science and Pollution Research*, *23*(1), 918–926. https://doi.org/10.1007/s11356-015-5650-9

Karim, M., Zhao, W., Rowley, D., Nelson, D., & Gomez-Chiarri, M. (2013). Probiotic strains for shellfish aquaculture: Protection of eastern oyster, *Crassostrea virginica*, larvae and juveniles against bacterial challenge. *Journal of Shellfish Research*, *32*(2), 401–408. https://doi.org/10.2983/035.032.0220

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

Kolndadacha, O. D., Adikwu, I. A., Okaeme, A. N., Atiribom, R. Y., Mohammed, A., & Musa, Y. M. (2011). The role of probiotics in aquaculture in Nigeria-a review. *Continental Journal of Fisheries and Aquatic Sciences*, 5(1), 8-15.

Lacoste A, Jalabert F, Malham S, Cueff A, Gélébart F, Cordevant C, Lange M, Poulet SA. (2001). A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Diseases of aquatic organisms*, 46(2), 139-45 doi: 10.3354/dao046139. PMID: 11678229.

Lulijwa, R., Rupia, E. J., & Alfaro, A. C. (2020). Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. In *Reviews in Aquaculture*, 12(2), 640–663. Wiley-Blackwell. https://doi.org/10.1111/raq.12344

Lupo, C., Travers, M. A., Tourbiez, D., Barthélémy, C. F., Beaunée, G., & Ezanno, P. (2019). Modeling the transmission of *Vibrio aestuarianus* in pacific oysters using experimental infection data. *Frontiers in Veterinary Science*, 6, 142. https://doi.org/10.3389/fvets.2019.00142

Luu, Q. H., Nguyen, T. B. T., Nguyen, T. L. A., Do, T. T. T., Dao, T. H. T., & Padungtod, P. (2021). Antibiotics use in fish and shrimp farms in Vietnam. *Aquaculture Reports*, 20. https://doi.org/10.1016/j.aqrep.2021.100711

Meng, J., Zhang, L., Huang, B., Li, L., & Zhang, G. (2015). Comparative analysis of oyster (*Crassostrea gigas*) immune responses under challenge by different *Vibrio* strains and conditions. *Molluscan Research*, *35*(1), 1–11. https://doi.org/10.1080/13235818.2014.919696

Miranda, C. D., Godoy, F. A., & Lee, M. R. (2018). Current status of the use of antibiotics and the antimicrobial resistance in the chilean salmon farms. In *Frontiers in Microbiology*, 9. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.01284

Murray, A. G., & Peeler, E. J. (2005). A framework for understanding the potential for emerging diseases in aquaculture. *Preventive Veterinary Medicine*, *67*, 223–235. https://doi.org/10.1016/j.prevetmed.2004.10.012

Nimalan, N., Sørensen, S.L., Fečkaninová, A., Koščová, J., Mudroňová, D., Gancarčíková, S., Vatsos, I.N., Bisa, S., Kiron, V. and Sørensen, M. (2023). Supplementation of lactic acid bacteria has positive effects on the mucosal health of Atlantic salmon (*Salmo salar*) fed soybean meal. *Aquaculture Reports*, 28, 101461.

Pernet, F., Lupo, C., Bacher, C., & Whittington, R. J. (2016). Infectious diseases in oyster aquaculture require a new integrated approach. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1689). https://doi.org/10.1098/rstb.2015.0213

Prado, S., Montes, J., Romalde, J. L., & Barja, J. L. (2009). Inhibitory activity of Phaeobacter strains against aquaculture pathogenic bacteria. *International Microbiology*, *12*(2), 107–114. https://doi.org/10.2436/20.1501.01.87

Prado, S., Romalde, J. L., & Barja, J. L. (2010). Review of probiotics for use in bivalve hatcheries. *Veterinary Microbiology*, *145*(3–4), 187–197. https://doi.org/10.1016/j.vetmic.2010.08.021

Richards, G. P., Watson, M. A., Needleman, D. S., Church, K. M., & Häse, C. C. (2015). Mortalities of Eastern And Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Applied and Environmental Microbiology*, 81(1), 292–297. https://doi.org/10.1128/AEM.02930-14

Siboni, N., King, W. L., Williams, N. L. R., Scanes, E., Giardina, M., Green, T. J., Ostrowski, M., O'Connor, W., Dove, M., Labbate, M., & Seymour, J. R. (2024). Increased abundance of potentially pathogenic *Vibrio* and a marine heatwave co-occur with a Pacific Oyster summer mortality event. *Aquaculture*, 583. https://doi.org/10.1016/j.aquaculture.2024.740618

Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., Vlak, J. M., Jones, B., Morado, F., Moss, S., Lotz, J., Bartholomay, L., Behringer, D. C., Hauton, C., & Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology*, *110*(2), 141–157. https://doi.org/10.1016/j.jip.2012.03.013

Stentiford, G. D., Sritunyalucksana, K., Flegel, T. W., Williams, B. A. P., Withyachumnarnkul, B., Itsathitphaisarn, O., & Bass, D. (2017). New Paradigms to Help Solve the Global Aquaculture Disease Crisis. *PLoS Pathogens*, *13*(2). https://doi.org/10.1371/journal.ppat.1006160

Sugumar G, Nakai T, Hirata Y, Matsubara D, Muroga K. *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. *Dis Aquat Organ*. 33(2)., 111-118. doi: 10.3354/dao033111. PMID: 9684317.

Travers, M. A., Tourbiez, D., Parizadeh, L., Haffner, P., Kozic-Djellouli, A., Aboubaker, M., Koken, M., Dégremont, L., & Lupo, C. (2017). Several strains, one disease: Experimental investigation of *Vibrio aestuarianus* infection parameters in the Pacific oyster, *Crassostrea gigas*. *Veterinary Research*, *48*(1). https://doi.org/10.1186/s13567-017-0438-1

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, *4*(1). https://doi.org/10.1186/s42523-022-00186-9

Ushijima, B., Richards, G. P., Watson, M. A., Schubiger, C. B., & Häse, C. C. (2018). Factors affecting infection of corals and larval oysters by *Vibrio coralliilyticus*. *PLoS ONE*, *13*(6). https://doi.org/10.1371/journal.pone.0199475

Vezzulli, L., Pezzati, E., Stauder, M., Stagnaro, L., Venier, P., & Pruzzo, C. (2015). Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. *Environmental Microbiology*, *17*(4), 1065–1080. https://doi.org/10.1111/1462-2920.12484

Yeh, H., Skubel, S. A., Patel, H., Cai Shi, D., Bushek, D., & Chikindas, M. L. (2020). From Farm to Fingers: an Exploration of Probiotics for Oysters, from Production to Human Consumption. In *Probiotics and Antimicrobial Proteins*, 12(2), 351–364. Springer. https://doi.org/10.1007/s12602-019-09629-3

Zhao, C., Men, X., Dang, Y., Zhou, Y., & Ren, Y. (2023). Probiotics Mediate Intestinal Microbiome and Microbiota-Derived Metabolites Regulating the Growth and Immunity of Rainbow Trout (*Oncorhynchus mykiss*). *Microbiology Spectrum*, 11(2). https://doi.org/10.1128/spectrum.03980-22

Chapter six: General discussion



Photograph taken by Kallen Sullivan

Introduction:

Oyster aquaculture provides a sustainable source of global protein, contributing to both food security and ecological restoration goals. Securing the future of this industry is largely dependent upon larval health, which when compromised halts the production of seedstock from hatcheries. The link between the larval microbiome and host health has been previously established, with a state of microbial dysbiosis (imbalance of the microbiome often instated by pathogenic microorganisms), causing large-scale oyster mortality events (Cram et al, 2023; Destoumieux-Garzón et al, 2024; Takyi et al, 2024; Vignier et al, 2021). Investigating this relationship further has significant potential to improve the ways in which oysters are produced and ensure the security of aquaculture.

This thesis therefore aims to contribute to the current understanding of the oyster larval microbiome, and investigate novel methods of its manipulation. I comprehensively address this by studying microbiomes in a range of different environments (wild, commercial and research) and two different oyster species (*Crassostrea gigas* and *Ostrea edulis).* Of these two oyster species, *C. gigas* is primarily farmed as a food source, whereas as the 'ecosystem engineer' *O. edulis* is cultivated for applications for both aquaculture and ecological restoration projects. I then attempted methods of microbial manipulation throughout chapters four and five as possible alternatives to antibiotic usage.

This chapter provides a broad overview of each experimental chapter and discusses key themes throughout the thesis such as: a lack of species level resolution from sequence data (Chapter one), alpha diversity trends between chapters (Chapter four), discussion of within-host vs environmental manipulation methods (Chapter five) and a common recommendation for future research – investigation of microbial functionality (future perspectives).

* * *

Chapter two: first investigation of the environmental microbiome throughout a wild bivalve mollusc spawning event.

This chapter describes the environmental microbiome throughout a wild spawning of *O. edulis*. This is the first piece of research to investigate the microbiome of a wild oyster spawning event, and is among the few studies to examine the *O. edulis* microbiome alongside Juste-Poinapen et al, 2024 and Wegner et al, 2025. This aim was achieved in collaboration with St Abbs Marine Station and Heriot Watt University, who collected water samples throughout a natural *O. edulis* spawning event at Loch Ryan UK (Chapman et al, 2019). Water samples were collected on four different days, three different depths within the water column and from eight different locations across the Loch, before being used to conduct larval counts. Full length 16S rRNA sequencing then allowed for the reporting of bacterial ID and abundance data from these samples.

It was originally hypothesised that larval abundance would be correlated with specific microbial signatures, however these were not detected. Microbial differences between water samples were instead most closely correlated with date, and not larval abundance, depth, or location of sample collection. These microbial differences were not driven by variations in species richness, but by the abundance of certain taxa. Most notably, the relative abundance of OTUs belonging to the *Rhodobacteraceae* family decreased as the spawning season progressed. With very few existing *O. edulis* microbiome studies, a comparison to the existing literature is fairly limited. Most relevantly, Juste-Poinapen et al, 2024 investigates the environmental microbiome of *O. edulis* reefs in the Dutch North Sea. Notably, samples were not collected throughout a spawning event, but from adult oyster shells, seawater and sediment. As was also reported within the Loch Ryan study, the majority of OTUs were also assigned to *Protobacteria* and *Bacteroidetes* phyla. Juste-Poinapen et al, 2024 did however report an oyster-associated microbiome, characterised by the presence of Archaea and close association to the sediment microbiome. This study along with chapter two of this thesis provide unique baseline microbial data of *O. edulis.* Together, they are advantageous in covering a wider range of life stages and habitat locations of this key restoration species.

Inverse abundance profiles of *Vibrio* and *Rhodobacteraceae* were also observed throughout the Loch Ryan spawning event. The importance of the *Vibrio* genus is frequently described

throughout this thesis, with species such as *Vibrio aestuarianus*, *Vibrio splendidus, Vibrio coralliilyticus* and *Vibrio tubiashii* being key oyster pathogens (Bean et al, 2024; Richards et al, 2015; Vezzulli et al, 2015; Wang et al, 2021). Since bacterial identification was not achieved at a species level, it is inconclusive as to whether the *Vibrio* OTUs detected included such pathogenic species. This provides one example of how future studies could be improved, where increased specificity would allow for greater functional insight of the microbiome in question. It should also be noted that larval settlement rates from this spawning event were not recorded, meaning that the microbial data cannot be assumed to be representative of successful settlement. Future studies may also therefore benefit from longer term monitoring of spawning events in this location along with collection of settlement rate data.

There is not sufficient evidence from this chapter to conclude that the inverse abundance profiles of *Vibrio* and *Rhodobacteraceae* were directly correlated. However, upon exploring the existing literature, this finding allowed for the formation of the hypothesis that the *Rhodobacteraceae* family may have important applications of bacterial disease control. This was formed in light of the knowledge that some species of the *Rhodobacteraceae* family produce the vibriostatic compound TDA (tropodithietic acid) (Geng et al, 2008). This hypothesis was then investigated through *in vivo* and *in vitro* experiments presented in chapter five.

Long-read sequencing was used within this chapter in order to increase the resolution level of the sequence data. Unfortunately, species level identification was not achieved – a theme also seen throughout chapters three and four. This is most likely attributed to the underdevelopment of taxonomy databases – where marine bacterial species are under characterised in contrast to other areas of microbial research such as the human gut. Should data have been reported at a species level, general data trends (alpha and beta diversity) are not expected to have been significantly different to the data currently presented. Species-level knowledge would however have been advantageous in possibly reducing the proportions of 'uncultured' taxa reported and providing a greater insight into the functional roles of OTUs identified.

Overall, this chapter provides important baseline information about the spawning environment of *O. edulis*. This not only contributes to the general understanding of the larval

microbiome, but is a prerequisite needed in order to develop microbiome manipulation strategies for the improvement of larval health.

* * *

Chapter three: a microbiome investigation throughout different spawning events of UK oyster hatcheries:

This chapter investigates the relationship between the microbiome of oyster larvae and their hatchery environment, with a particular focus on commercial oyster production. The majority of existing larval microbiome studies have been conducted in research-focused environments which are generally far more sterile than commercial hatchery settings (King et al, 2019; Lokmer and Wegner, 2015, Scanes et al, 2021a; Unzueta-Martínez et al, 2022a, Unzueta-Martinez et al, 2022b). There is therefore a need to carry out microbial research directly within the environment in which oysters are cultivated, where the data is more representative of commercial production. In particular, this information will be crucial in the application of microbiome-based research for making improvements in industry. This chapter follows oyster spawning events of *O. edulis* and *C. gigas* at two UK hatcheries (Portsmouth University and Morecambe Bay oyster hatchery). Three key aims were investigated:

1. Identification of core larval taxa from both hatcheries and their changes over time.
2. Comparison of larval bacterial profiles to those of their hatchery environments.
3. Investigate how hatchery processes specific to these locations (such as water filtration and algal production) influence microbial communities.

Extensive sampling procedures were carried out at each location, including sample types of larvae, larval culture water, hatchery water, biofilm (Morecambe Bay), pallial cavity water (Portsmouth), algae and axenic starter cultures. *This allowed the microbiome of the hatchery environment to be investigated comprehensively.* This study was limited by both practicality of sampling and the cost of sequencing. Future work should aim to expand on these sample

sets further still, by including biological replication, sequencing of samples collected daily throughout larval production and following larval development past settlement.

Core microbial taxa were successfully identified from larvae at both hatcheries. Importantly, these were more closely clustered closer to the developmental stage of larvae and not the location from which they were collected (upwelling or larval collection tray number). At both hatcheries, larvae in the later developmental stages clustered with fewer core larval taxa than those at earlier developmental stages. It is hypothesised that the core taxa identified at these hatcheries play important functional roles within their host and are therefore required in order to maintain larval health. Future studies should aim to characterise the functional roles of these core taxa, investigate the energetic costs associated with maintaining a core microbiome, and determine the extent of variability in the core microbiome composition among healthy larvae both within between spawning events.

A reduction in species richness was reported as larvae at Portsmouth progressed in their development. This may provide evidence that as larvae display selective control of their microbiome with age, possibly being less easily influenced by environmental microbiota, or it may be a direct response to the practice of pooling and splitting groups of larvae. An association between larval development and microbial variation has previously been reported by Unzueta-Martínez et al, 2022b in the Sydney Rock oyster (*Saccostrea glomerata).* Here later developmental stages were dominated by orders such as Vibrionales*,* Cellvibrionales and Rhizobiales which were present in very low relative abundances or not at all within the surrounding seawater. Defining the core larval microbiome is beneficial in identifying bacteria which are likely to play a functional role in oyster health. This is vital information needed to better understand larval processes such as development, metabolism and pathogen defence. An association has been reported between host microbiome diversity and complexity of the immune system in invertebrates with innate immune systems, however this relationship is yet to be investigated in oyster species (Timmins-Schiffman et al, 2021). An important area of future research would involve expanding upon such work to investigate the functional roles of core larval taxa in more depth.

Comparing the larval microbiome to that of their environment is largely limited to samples of larval culture water (Arfken et al, 2021; Asmani et al, 2016; Unzueta-Martínez et al, 2022b). Again, this chapter includes a broader range of sample types to more comprehensively report

on the hatchery microbiome. Existing studies are in agreement that larval microbiomes are distinct to that of their culture water, with microbial differences becoming more apparent during the later developmental stages (Unzueta-Martínez et al, 2022b). Similarly, in this chapter, microbiomes of larvae were found to be distinct to those of other sample types at both hatcheries. This suggests that the larval microbiome is not merely a reflection its environment, but that larvae impart some level of microbial selection themselves.

With the exception of Laroche et al, 2018, the effectiveness of biosecurity measures within oyster hatcheries is under researched. This chapter therefore aimed to fulfil this knowledge gap, reporting water filtration techniques at both hatcheries were concluded to be effective in reducing microbial diversity levels. Confirmation of such successful practices provides immediately useful information to these hatcheries, allowing them to better determine possible sources of microbial contamination to larval cultures. Water samples collected pre-UV treatment at Morecambe Bay showed a higher relative abundance of Nitrospinota, Verrucomicrobiota, Dependentiae, and Desulfobacterota than water collected post UV treatment. Laroche et al, 2018 suggests optimal UV treatment at 200 mJoules/cm^2 to reduce the species richness of seawater with no detrimental effect of this being observed in *C. gigas* larvae. Investigating the hatchery microbiome is therefore able to better inform hatchery biosecurity measures, limiting the transfer of pathogenic microorganisms to larval cultures. With hindsight, it would be worth adding overall microbial quantifications to each of these samples to help with interpretation of the relative quantification results inter-sample. Other hatchery rearing parameters such as larval density have the potential to influence the larval microbiome, however these were not reported throughout this chapter. If microbial taxa are assumed to be easily transferred between larvae, then it could be hypothesised that rearing dense cultures of larvae poses the risk of pathogen-transfer to entire stocks. This hypothesis has not been addressed within the current literature, and so may be a possible area for future study.

In conclusion, this chapter provides broad-scale microbial data which improves our understanding of the link between oyster larvae and their hatchery environment. Whilst similar aims have been investigated (Arfken et al, 2021; Asmani et al, 2016; Unzueta-Martínez et al, 2022a; Unzueta-Martínez et al, 2022b), this chapter addresses them within the context

of commercial production. This provides a dataset which can immediately advise on hatchery biosecurity measures and is more directly applicable to the improvement of the industry.

* * *

Chapter four: manipulation of the C. gigas larval microbiome via temperature and diet:

This chapter moves away from previous aims of collecting baseline microbial data, and instead investigates possible methods of microbial manipulation. The overuse and misuse of antibiotics in aquaculture threatens the sustainability of the industry, creating a demand for alternative methods of microbiome manipulation. This is ultimately for the objective of increased seedstock production in hatcheries via the general improvement of larval health or minimisation of disease events. This chapter therefore investigated two possible methods of microbiome manipulation, based around varying diet and treatment groups. In collaboration with Cefas, these aims were tested using culture dependent and independent techniques.

An investigation of microbial manipulation via temperature control was conducted under the rationale that culturing *C. gigas* larvae at 25˚C would result in a larval microbiome that had a higher relative abundance of pathogenic bacteria than if larvae were cultured at 20˚C. Larvae grown at 25˚C exhibited a faster growth rate, but similar culturable bacterial load to those grown at 20˚C. In order to fully conclude this aim, identification of the bacterial genera within the larval microbiomes was required (allowing for the identification of possibly pathogenic groups). This was unfortunately not achieved, due to insufficient 16S rRNA amplification. In order for future experiments to collect this data, changes to the experimental set up are recommended, mainly comprising of increasing the larval culture volumes from 1 l to 15 l. This increase in volume would allow for more larvae per sample to be collected, and with more biomass available, successful 16S rRNA amplification would be more likely. The association between temperature and the oyster microbiome is most frequently investigated within the context of climate change. For example, elevated seawater temperatures increase species richness of the adult Sydney Rock oyster haemolymph microbiome (Scanes et al, 2021b). Existing literature therefore indicates that similar effects may be seen in larvae of *C.*

gigas. Repetition of the temperature experiment presented in this chapter to include 16S sequence data would therefore be worthwhile, with the potential to better inform hatchery practices.

The majority of this chapter investigates dietary control as a method of microbial manipulation. Larvae (*C. gigas)* were strip spawned and reared for 15 days under either a control diet (fed *Tisochrysis lutea*) or experimental diet (fed *Tisochrysis lutea* with *Tetraselmis suecica*). Based on anecdotal information from oyster hatcheries, it was hypothesised larvae fed the experimental diet would exhibit an increased growth rate in comparison to the control group. This was based on the rationale that although *Tetraselmis suecica* cells are too large to be ingested by the larvae, that the addition of this algae to the water beneficially alters the larval microbiome resulting in an increased growth rate. Larvae fed with the experimental diet did display an improved growth rate in comparison the control group. Sequence data concluded that microbial differences were statistically significant between the two algal species, driven not by species richness but by the abundance of taxa belonging to Actinobacteria, Bdellovibrionota and Bacilli. Understanding the functional roles of these bacterial groups is needed in order to fully conclude if these differences in microbial taxa were responsible for the increased growth rates seen in the experimental group. Future research may also consider the effect *Tetraselmis suecica* to seawater chemistry, and whether this created optimal growth conditions for larvae under this treatment.

This chapter reported a lower species richness in 14-day old larvae treated with the experimental diet vs those treated with the control diet. Since larvae fed the experimental diet exhibited an increased growth rate, this decrease in species diversity is not thought to have been detrimental to larval health. A decrease in species richness (although not statistically significant), was also observed in Chapter three – within *O. edulis* larvae over time. Again, since these cultures were observed to be in good health, a decline in species richness is unlikely to be linked to a decline in larval health. It is however possible that larval mortalities may occur should the number of observed features or average Chao1 indices fall below a certain threshold value. Determining this threshold value alongside routine microbial testing of larval cultures may be advantageous in providing early warning signs of mass mortality events.

Overall, this chapter concludes that algal feed do have an influence over the larval microbiome with the majority of larval OTUs also being found in corresponding algae. Determining influencing factors of the larval microbiome is essential to understand environmental conditions and rearing practices effect larval heath and development.

* * *

Chapter five: Exploring the Rhodobacteraceae family for inhibitory effects against Vibrio infection in Crassostrea gigas.

This chapter shares a primary aim with chapter four – investigation of larval microbial manipulation methods. However in contrast, this chapter utilises within-host microbiome-intervention based methods instead of the exposure, or environmental methods described throughout chapter four. Both mechanisms have the potential to improve larval health and the success of oyster cultivation and would ideally be used in tangent. Manipulation via environmental parameters may be more likely to provide general health benefits to the larvae such as an increased feeding rates. These methods may be easier to apply at a system-level, but are less likely to confer resistance against specific pathogens. This is an issue which can be better addressed through the development of within-host manipulation methods (as addressed throughout this chapter). Decreasing the susceptibility of larvae to particular pathogens independently of the environment may reduce the pressure on maintaining strict environmental conditions within the hatchery. Chapter five therefore investigates one possible method of method of within-host microbial manipulation.

The *Vibrio* genus of bacteria is of particular relevance to the oyster microbiome, with species such as *Vibrio aestuarianus* (*V. aestuarianus*) and *Vibrio splendidus* (*V. splendidus*) causing oyster mortalities in both wild and farmed scenarios (Bean et al, 2024; Dubert et al, 2016; Sugumar et al, 1998; Lacoste et al, 2001). Whilst the use of antibiotics provides one possible solution to limiting these bacterial infections, they are not a sustainable solution (Bhat and Altinok, 2023; Bondad-Reantaso et al, 2023; Caputo et al, 2023). Probiotic treatments offer one particular alternative to antibiotic usage. These are however far more developed for

other aquaculture species such as salmon, seabass and Pacific Whiteleg shrimp than they are for the bivalve industry (El-Saadony et al, 2021; Hai et al, 2015). This chapter therefore seeks to address this knowledge gap by investigating strains of the Rhodobacteraceae family for inhibitory effects against *Vibrio* infection in *C. gigas*.

As previously discussed, this chapter explores a hypothesis formed as a continuation from a finding within chapter two. Strains of Rhodobacteraceae were tested for inhibitory effects against *V. aestuarianus* and *V. splendidus*. These strains belonged to genera of *Sulfitobacter, Marivita, Jannaschia* and *Oceanibulbus* which are not amongst current shellfish probiotic treatments (Ringø et al, 2020). Novel in vitro agar plate assays were initially used to select for the Rhodobacteraceae strains which showed the most inhibitory potential against *V. splendidus*. These strains were then used in vivo, in bath challenge experiments with adult *C. gigas* individuals challenged with either *V. aestuarianus* or *V. splendidus.* Most importantly, two candidate Rhodobacteraceae strains improved survival rate from 0% to 50% and 60% following *V. aestuarianus* infection. This is promising preliminary data which suggests that these Rhodobacteraceae strains (named as numbers 1159 and 1572) would be suitable candidates for further probiotic testing. Crucially, further work is required to replicate these results. Initially, this would require repetition of the bath challenges presented throughout this chapter, using larger sample sets of oysters. If successful, larger-scale trials could then be conducted with oyster hatcheries – ideally incorporating a range of different oyster species and life stages. This chapter contributes towards the development of antibiotic alternatives – a theme of research which has great potential to improve the sustainability and security of aquaculture.

* * *

Future perspectives and research recommendations:

This thesis has improved the current understanding of the oyster microbiome and ways in which this knowledge can be used for the advancement of shellfish farming. However, it also helps to pinpoint knowledge gaps that can direct future research into the oyster microbiome.

For example, current literature is heavily focused upon the reporting of prokaryotes of the oyster microbiome. There is however there is also a clear need to understand eukaryotic and viral communities. This is with the understanding that disease events in oyster hatcheries (and in aquaculture generally) are driven by complex interactions between microorganisms, host immunity and their external environment (Bass et al, 2019). Gaining a more comprehensive understanding of the oyster microbiome and the interactions which occur within it would most likely provide important information about oyster health and responses during disease events. More recent research has been beneficial in beginning to address this knowledge gap (Wegner et al, 2025; Zhong et al, 2024). A state of parasitic infection in both *O. edulis* and *C. gigas* can be more accurately predicted by the prokaryotic than eukaryotic microbiota present (Wegner et al, 2025). This is however not to say that eukaryotic communities should not be studied, with protists of the *Labyrinthula, Aplanochytrium* genera being detected consistently within the *C. gigas* microbiome throughout simulated ocean warming and acidification experiments (Zhong et al 2024). These protists are therefore likely to play an important functional role within the *C. gigas* microbiome, particularly in response to climate change stressors. This highlights another reoccurring theme for future research - the need to investigate functional roles of core microbial taxa.

Chapter three of this thesis, along with previous studies have identified core microbial taxa throughout a range of different oyster species and life stages (Arfken et al, 2021; Cho et al, 2024; Unzueta-Martínez et al, 2022). This information identifies taxa e.g. those belonging to Rhodobacteraceae, Actinobacteria, Bacteroidota, *Labyrinthula* and *Aplanochytrium*, which are most likely to play important functional roles in oyster health. These functional roles may be centred around larval growth, development, environmental adaptation or immune system priming. Investigations into these specific roles and their mechanisms would greatly improve the current understanding of bivalve biology, with possible applications in enhancing larval growth and survival rates or targeted disease prevention. In order to identify these functional roles, future studies may initially base research aims around the isolation and culture these core bacterial taxa. In order to do this, identification of the bacteria at a species level would firstly be required. The use of whole genome sequencing (WGS) such as shotgun sequencing is therefore be advised to achieve this. Initial in vitro experiments (such as those presented throughout Chapter five), would then select the most appropriate candidates for

metagenomic, metabolic or transcriptomic techniques to be applied. Future studies would be advised to isolate and test as many bacterial species as possible, in order to improve the chances of identifying functional roles.

Understanding the functional roles of core microbial taxa would also aid creating more targeted approaches of microbial manipulation. This thesis highlights the need for alternative treatments to antibiotics to secure the sustainability of shellfish production. This may be achieved by the transfer of probiotic and prebiotic treatments which are better developed within finfish aquaculture, into bivalve species (Hai et al, 2015). Alternatively, future research may utilise mechanisms such as quorum quenching. This is the disruption of the cell-to-cell communications that regulate virulence potential in bacteria (Shaheer et al, 2021). This method has been investigated for the control of *Vibrio harveyi* and *Vibrio parahaemolyticus* infection in shrimp and seabass aquaculture, and is thought to result in less selective pressure than antibiotic usage (Ghanei-Motlagh et al, 2021; Shaheer et al, Sun et al, 2022; 2021; Zhang et al, 2023). Whilst this is a promising strategy for controlling pathogenic bacteria which directly affect oyster cultivation, there are no trials to date which apply these methods within oyster hatcheries. This is therefore another recommendation for future research, where trials should initially be focused on the larvae of *C. gigas* for increased seedstock production from hatcheries.

Chapter three of this thesis highlights the needs to study the oyster microbiome during commercial production – a process significantly limited by larval disease events. Microbial investigations at a commercial scale during disease events would allow for important comparisons to be made between successful and unsuccessful hatchery production runs. These disease events are however unpredictable, making sample collection difficult. Maintaining positive relationships and communication between industry and academia is therefore vital in order to conduct research within hatchery settings. This may be achieved through a number of different ways, such as collaboration at public engagement events, e.g. the annual Stranraer oyster festival or Royal Highland Show (Scotland, UK). This would increase communication between academia and industry whilst working towards a mutually beneficial goal of raising public awareness around the importance of oyster farming. This strengthened relationship may in turn lead to more formal benefits such as regular meetings, joint grant applications or training and internship positions. Generally, this would facilitate

more research oppourtunities, covering a greater range of hatchery locations, oyster species and increase the changes of collecting microbial data during a crash event.

* * *

Final conclusions:

This thesis improves the current understanding of the oyster larval microbiome, covering key species of *O. edulis* and *C. gigas* in wild, farmed and experimental scenarios. Investigations of oyster larvae and their hatchery environment from two UK hatcheries aided in fulfilling a knowledge gap of microbial studies conducted during commercial production. A demand for alternative methods to antibiotic treatment was recognised, and methods of microbial manipulation via temperature, diet and probiotic control were investigated. Overall, this work contributes to the improvement of oyster aquaculture in the interest of securing food sustainability and ecological restoration goals.

References:

Bass, D., Stentiford, G. D., Wang, H. C., Koskella, B., & Tyler, C. R. (2019). The Pathobiome in Animal and Plant Diseases. In *Trends in Ecology and Evolution*, 34 (11), 996–1008. Elsevier Ltd. https://doi.org/10.1016/j.tree.2019.07.012

Bean, T. P., Farley, H., Nascimento-Schulze, J., & Regan, T. (2024). Scottish oyster mortality event and association with *Vibrio aestuarianus*. *Aquaculture Reports*, *39*. https://doi.org/10.1016/j.aqrep.2024.102480

Bhat, R. A. H., & Altinok, I. (2023). Antimicrobial Resistance (AMR) and Alternative Strategies for Combating AMR in Aquaculture. *Turkish Journal of Fisheries and Aquatic Sciences*, *23*(11). https://doi.org/10.4194/trjfas24068

Bondad-Reantaso, M. G., MacKinnon, B., Karunasagar, I., Fridman, S., Alday-Sanz, V., Brun, E., Le Groumellec, M., Li, A., Surachetpong, W., Karunasagar, I., Hao, B., Dall'Occo, A., Urbani, R., & Caputo, A. (2023). Review of alternatives to antibiotic use in aquaculture. In *Reviews in Aquaculture*, 15, (4), 1421–1451. John Wiley and Sons Inc. https://doi.org/10.1111/raq.12786

Caputo, A., Bondad-Reantaso, M. G., Karunasagar, I., Hao, B., Gaunt, P., Verner-Jeffreys, D., Fridman, S., & Dorado-Garcia, A. (2023). Antimicrobial resistance in aquaculture: A global analysis of literature and national action plans. In *Reviews in Aquaculture*, 15, (2), 568–578. John Wiley and Sons Inc. https://doi.org/10.1111/raq.12741

Chapman, E. C., Rodriguez-Perez, A., Hugh-Jones, T., Bromley, C., James, M. A., Diele, K., & Sanderson, W. G. (2021). Optimising recruitment in habitat creation for the native European oyster (*Ostrea edulis*): implications of temporal and spatial variability in larval abundance. *Marine Pollution Bulletin*, 170, 112579.

Cram, J. A., McCarty, A. J., Willey, S. M., & Alexander, S. T. (2024). Microbial community structure variability over the development of healthy and underperforming oyster larval hatchery broods. *Frontiers in Aquaculture*, *3*. https://doi.org/10.3389/faquc.2024.1427405

Destoumieux-Garzón, D., Montagnani, C., Dantan, L., Nicolas, N. D. S., Travers, M. A., Duperret, L., Charrière, G. M., Toulza, E., Mitta, G., Cosseau, C., & Escoubas, J. M. (2024). Cross-talk and mutual shaping between the immune system and the microbiota during an oyster's life. In *Philosophical Transactions of the Royal Society B: Biological Sciences*, 379, (1901). Royal Society Publishing. https://doi.org/10.1098/rstb.2023.0065

Dubert, J., Osorio, C. R., Prado, S., & Barja, J. L. (2016). Persistence of Antibiotic Resistant *Vibrio spp*. in Shellfish Hatchery Environment. *Microbial Ecology*, *72*(4), 851–860. https://doi.org/10.1007/s00248-015-0705-5

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A. O., Dhama, K., & Abdel-Latif, H. M. R. (2021). The functionality of probiotics in aquaculture: An overview. In *Fish and Shellfish Immunology*, 117, 36–52. Academic Press. https://doi.org/10.1016/j.fsi.2021.07.007

Geng, H., Bruhn, J. B., Nielsen, K. F., Gram, L., & Belas, R. (2008). Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. *Applied and Environmental Microbiology*, *74*(5), 1535–1545. https://doi.org/10.1128/AEM.02339-07

Ghanei-Motlagh, R., Mohammadian, T., Gharibi, D., Khosravi, M., Mahmoudi, E., Zarea, M., El-Matbouli, M., & Menanteau-Ledouble, S. (2021). Quorum quenching probiotics modulated digestive enzymes activity, growth performance, gut microflora, haemato-biochemical parameters and resistance against *Vibrio harveyi* in Asian seabass (*Lates calcarifer*). *Aquaculture*, *531*. https://doi.org/10.1016/j.aquaculture.2020.735874

Hai, N. V. (2015). The use of probiotics in aquaculture. In *Journal of Applied Microbiology*, 119, (4), 917–935. https://doi.org/10.1111/jam.12886

Juste-Poinapen, N. M. S., Lu, Y., De Hoyos, B. B., Birch, G. C., & Saurel, C. (2024). Characterisation of the Bacteria and Archaea Community Associated with Wild Oysters, at Three Possible Restoration Sites in the North Sea. *Open Journal of Marine Science*, *14*(02), 19–40. https://doi.org/10.4236/ojms.2024.142002

King, W. L., Jenkins, C., Go, J., Siboni, N., Seymour, J. R., & Labbate, M. (2019). Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. *Microbial Ecology*, *77*(2), 502–512. https://doi.org/10.1007/s00248-018-1226-9

Lokmer, A., & Mathias Wegner, K. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME Journal*, *9*(3), 670–682. https://doi.org/10.1038/ismej.2014.160

Richards, G. P., Watson, M. A., Needleman, D. S., Church, K. M., & Häse, C. C. (2015). Mortalities of Eastern And Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Applied and Environmental Microbiology*, *81*(1), 292–297. https://doi.org/10.1128/AEM.02930-14

Ringø, E. (2020). Probiotics in shellfish aquaculture. In *Aquaculture and Fisheries*, 5, (1), 1–27). KeAi Communications Co. https://doi.org/10.1016/j.aaf.2019.12.001

Scanes, E., Parker, L. M., Seymour, J. R., Siboni, N., Dove, M. C., O'Connor, W. A., & Ross, P. M. (2021a). Microbiomes of an oyster are shaped by metabolism and environment. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-00590-2

Scanes, E., Parker, L. M., Seymour, J. R., Siboni, N., King, W. L., Danckert, N. P., Wegner, K. M., Dove, M. C., O'Connor, W. A., & Ross, P. M. (2021b). Climate change alters the haemolymph microbiome of oysters. *Marine Pollution Bulletin*, *164*(August 2020), 111991. https://doi.org/10.1016/j.marpolbul.2021.111991

Sun, X., Liu, J., Deng, S., Li, R., Lv, W., Zhou, S., Tang, X., Sun, Y. Z., Ke, M., & Wang, K. (2022). Quorum Quenching Bacteria *Bacillus velezensis* DH82 on Biological Control of *Vibrio parahaemolyticus* for Sustainable Aquaculture of *Litopenaeus vannamei*. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.780055

Takyi, E., Stevick, R. J., Witkop, E. M., Gregg, L., Chesler-Poole, A., Small, J. M., White, M. M., Hudson, R., Giray, C., Rowley, D. C., Nelson, D. R., & Gomez-Chiarri, M. (2024). Probiotic treatment modulates the bacterial microbiome of larval eastern oysters, *Crassostrea virginica*, in hatcheries. *Aquaculture*, *583*. https://doi.org/10.1016/j.aquaculture.2024.740624

Timmins-Schiffman, E., White, S. J., Thompson, R. E., Vadopalas, B., Eudeline, B., Nunn, B. L., & Roberts, S. B. (2021). Coupled microbiome analyses highlights relative functional roles of bacteria in a bivalve hatchery. *Environmental Microbiomes*, *16*(1). https://doi.org/10.1186/s40793-021-00376-z

Unzueta-Martínez, A. (2022a). Determining the Composition of Resident and Transient Members of the Oyster Microbiome. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.828692

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022b). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, *4*(1). https://doi.org/10.1186/s42523-022-00186-9

Vezzulli, L., Pezzati, E., Stauder, M., Stagnaro, L., Venier, P., & Pruzzo, C. (2015). Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. *Environmental Microbiology*, *17*(4), 1065–1080. https://doi.org/10.1111/1462-2920.12484

Vignier, J., Laroche, O., Rolton, A., Wadsworth, P., Kumanan, K., Trochel, B., Pochon, X., & King, N. (2021). Dietary Exposure of Pacific Oyster (*Crassostrea gigas*) Larvae to Compromised Microalgae Results in Impaired Fitness and Microbiome Shift. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.706214

Wang, H., Yang, B., Li, X., Li, Q., & Liu, S. (2021). Screening of bacterial pathogens associated with mass summer mortality of the Pacific oyster, *Crassostrea gigas*, in China. *Aquaculture Reports*, *20*. https://doi.org/10.1016/j.aqrep.2021.100672

Wegner, K. M., Morga, B., Guillou, L., Strittmatter, M., Lecadet, C., Travers, M. A., Tourbiez, D., Gervais, O., & Arzul, I. (2025). Prokaryotic microbiota outperform eukaryotic microbiota in differentiating between infection states of iconic diseases of two commercial oyster species. *Aquaculture*, *594*. https://doi.org/10.1016/j.aquaculture.2024.741363

Zhong, K. X., Chan, A. M., Collicutt, B., Daspe, M., Finke, J. F., Foss, M., Green, T. J., Harley, C. D. G., Hesketh, A. V., Miller, K. M., Otto, S. P., Rolheiser, K., Saunders, R., Sutherland, B. J. G., & Suttle, C. A. (2024). The prokaryotic and eukaryotic microbiome of Pacific oyster spat is shaped by ocean warming but not acidification. *Applied and Environmental Microbiology*, *90*(4). https://doi.org/10.1128/aem.00052-24

Appendices:

The following publication: 'Scottish oyster mortality event and association with *Vibrio aestuarianus*' is provided below as an appendices to this thesis. This paper was published in Aquaculture Reports in December 2024. As per the contribution statement, my contributions to this publication included reviewing and editing, methodology, investigation and formal analysis. More specifically, I carried out the qPCR assays for bacterial quantification and methods described for bacterial isolation.

This paper investigates a summer mortality event of *C. gigas* in Scotland, reporting the presence of *Vibrio aestuarianus* and *Vibrio splendidus* alongside the absence of oyster herpes virus (OsHV-1). In vivo experiments with *V. aestuarianus* resulted in significantly higher mortalities than with *V. splendidus* and were exacerbated by higher temperatures. The techniques for bacterial isolation described within this publication were necessary in order to grow the same species for use in chapter five of this thesis.

Scottish oyster mortality event and association with *Vibrio aestuarianus*

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Running page head: Vibrio associated Pacific oyster mortality

Key words: *Vibrio*; Pacific oyster; Mortality; Scotland; Aquaculture; Genome; Microbiology

Abstract

Pacific oysters, *Crassostrea* (*Magallana*) *gigas,* are the most commonly cultured invertebrate species globally. Recent years have seen outbreaks of summer mortality across the globe, mainly caused by viral and bacterial pathogens such as OsHV-1 µvar and *Vibrio* spp. Despite isolated outbreaks in southern England, the UK has remained largely free from these pathogens. A summer mortality event in Scotland was investigated after *Vibrio* spp. were implicated. We identified two key *Vibrio* species (*V. aestuarianus* and *V. splendidus*) but complete absence of OsHV-1. There was a high prevalence of *V. aestuarianus* among moribund and dead oysters, along with an increase in *V. aestuarianus* detected in the water column and sediment. Consistent with strains associated with oyster mortalities elsewhere in Scotland and mainland Europe, *V. aestuarianus* from this site belongs to Lineage A. Unlike *V. splendidus,* we could not culture *V. aestuarianus* from frozen tissue of infected oysters. *In vivo* infection experiments with *V. aestuarianus* resulted in significantly higher mortalities than with *V. splendidus* and were exacerbated by increased temperature. These findings suggest that mortalities are caused by *V. aestuarianus*, which may have been intensified by handling stress and rising temperatures.

1. Introduction

The Pacific oyster, *Crassostrea (Magallana) gigas,* is the most abundantly farmed mollusc species globally (FAO, 2024). This species also dominates UK oyster production (FAO, 2024), due to its rapid growth rate, adaptability to the farmed environment, and resistance to recalcitrant diseases such as Bonamiosis which impact native oyster (*Ostrea edulis*) production (Laing et al., 2006). The most common and significant challenge to Pacific oyster culture, however, remains mortality caused by infectious disease.

The UK oyster industry is based around many small-scale operations which enable production of a high-value and unique product (Regan et al., 2021), but these smaller businesses are generally highly susceptible to short-term shocks in production (Jennings et al., 2016). Due to their relative proximity and regular trade, farming sites throughout western Europe often experience similar infectious disease threats. Nonetheless, the European legislative framework as a whole delineates between nations and allows for some control of disease spread, usually from areas of highest production to areas on the geographical fringes of production. Scotland and north-east England continue to remain largely free from diseases such as Pacific oyster mortality syndrome, or POMS, caused by pathogens such as Ostreid Herpesvirus 1 (OsHV-1) in conjunction with opportunistic pathogens often including *Vibrio* species of bacteria (de Lorgeril et al., 2018). Since the initial appearance of OsHV-1 μvar in France, the virus has been found in many oyster-producing regions globally (Dégremont et al., 2015; Renault et al., 2014) and infection can result in 100% mortality, with the highest impact usually observed in juvenile oysters (EFSA Panel on Animal Health & Welfare, 2010). However, resistance to OsHV-1 is a feature found in the genetics of many farming stocks (Divilov et al., 2023; Gutierrez et al., 2018) and breeding programmes producing stock with reduced

susceptibility to OsHV-1 are now commonplace (Dégremont et al., 2016; King et al., 2019). To date, there are no known methods for treatment or eradication of OsHV-1, and whilst promising, prevention through methods such as immune priming (Lafont et al., 2020) are yet to be used routinely. As such, areas naïve to the virus, such as Scotland, rely entirely on biosecurity to prevent spread, i.e. limiting movement of oysters from OsHV-1-positive sites to disease-free sites.

Vibrio bacterial species (*Vibrio* spp.) are almost ubiquitous in aquatic environments and filter feeding, sessile oysters are continuously exposed to an assortment of these organisms. Most *Vibrio* spp. are benign and *Vibrio* that do cause disease in bivalves tend not to affect humans and *vice versa* (Letchumanan et al., 2014). Rare, but significant summer mortality events caused by *Vibrio* spp. alone have been recorded in France since before 2000 (Lacoste et al., 2001) with various species reported to affect oysters at different stages of the life cycle including *V. coralliilyticus* and *V. neptunius* in larval animals, and *V. aestuarianus, V. anguillarum, V. crassostreae, V. splendidus*, and *V. tubiashii* (among others) infecting spat and adult animals (Dégremont et al., 2021). Since 2001 there have been numerous notable observations of mortality associated with the presence of *Vibrio aestuarianus* across Europe (Mesnil et al., 2023). The vast majority of the sites suffering from these events are also host to OsHV-1, which can make disease aetiology difficult to disentangle (Mesnil et al., 2023). One key indicator, contrary to classical OsHV-1 symptoms, is that adult *C. gigas* appear in general to be more susceptible than spat to mortality caused by *V. aestuarianus* infection (Sihvonen, 2015). More recently, there have also been multiple reports of mortalities in Ireland, in areas that are unaffected by OsHV-1, of animals close to harvest size dying through *V. aestuarianus* associated disease (Coyle et al., 2023). Unlike OsHV-1, no legislative measures are in place for

Vibrio spp., likely due to difficulties in unequivocally identifying infectious strains of this ubiquitous bacteria, as is generally required for control measures to be effective.

In summer 2019 a significant mortality event at a Scottish Pacific oyster farm was reported to the Fish Health Inspectorate. Initial investigations on mortality samples from this site reported strains of both *Vibrio splendidus* and *Vibrio aestuarianus,* but no OsHV-1. This manuscript describes assessment of mortalities from subsequent years, suggesting that this may be the first published report describing a recurring oyster summer mortality event in Scotland associated with pathogenic *Vibrio* spp*.* To investigate the cause of these mortalities in more depth, and clarify the potential impact of *Vibrio,* samples of water, sediment, and recently dead or moribund oysters were taken throughout the following year and assessed for the presence of key aetiological agents. Microbiome analysis was performed on selected mortality samples. Strains of *Vibrio* bacteria isolated from moribund samples were sequenced and used for *in vivo* infection experiments. Here we present the data identifying the likely aetiology as well as outlining some of the factors which may have roles to play in the process of disease progression.

2. Methods

2.1 Site parameters

The oyster farm is located within a sea loch on the west coast of Scotland (exact location not disclosed at farm owner's request). The site of roughly 7 ha produces animals from seed through to market size via a bag and trestle system with regular oyster grading and re-sorting into black mesh oyster bags (1 x 0.5 m). Accurate measurements of on-site water temperatures were done by temperature logger every 30 min, located approximately 60 cm

above chart datum within the oyster farm, and placed inside a white plastic container to avoid direct sunlight, then inside an oyster bag to accurately estimate water temperature inside the bags.

2.2 Sample collection

Samples were collected monthly during favourable tides from April to October of 2020, 2021 and 2022 (**Supplemental Table 1**). Four individual water and sediment samples and six live oyster samples were taken at low tide on each time-point. Oysters were taken from within at least two bags in the same oyster row each time. Sediment and water were taken from adjacent locations. Due to COVID-19-associated travel constraints, all material was frozen at -20 °C at the remote sampling site until transportation to the laboratory became possible. At the end of 2020 when restrictions had been lifted, the same process was used for all routine monitoring samples, but some extra samples were also transported chilled in an attempt to provide suitable material from which the detected *V. aestuarianus* strain could be isolated. Water samples were collected between oyster trestles by submerging sterile 50 ml falcon tubes just below the surface of the water, removing the cap to allow the vial to fill, then reapplying the lid before lifting the tube out of the water. Sediment samples were taken in four 50 ml falcon tubes, by using the tube to scoop roughly 40 to 50 ml of surface sediment from the upper 20 mm of sediment underneath oyster trestles. Any remaining water was poured off the top of the tube before sealing. Live oysters of harvest size (circa 10-15 cm shell height) were collected and frozen whole in zip-lock bags. When unusual mortality (i.e. over 10% within an oyster bag) was observed, dead or moribund oysters (open valves) with intact tissues were opportunistically sampled by removing remaining meat from up to a total of 10 animals into sterile 50 ml falcon tubes before freezing.

2.3 DNA extractions

Thawed water samples were thoroughly mixed by vortexing for 20 seconds. 15 ml of each was added to a 50 ml falcon tube containing 33 ml of ethanol and 1.5 ml of 3 M sodium acetate. After 1 hour incubation at -80 °C, tubes were centrifuged at 4°C, 1093 g rcf for 30 min to pellet any cellular material and free DNA. Supernatant was poured off and ethanol allowed to evaporate for 15 min. DNA was then extracted from 250 mg (±10%) of the pellet with the Powersoil DNA extraction kit (Qiagen) following manufacturer's protocols including optional incubation steps.

Thawed sediment samples were mixed thoroughly at room temperature to the point of being a homogeneous mixture by a combination of manual shaking and vortexing. 250 mg (±10%) of sediment was removed from each falcon tube and processed with the Powersoil DNA extraction kit (Qiagen) following manufacturers protocols including optional incubation steps.

Frozen harvest size whole oyster samples were partially thawed at room temperature, the top (left) valve removed with a sterile scalpel and approximately 200 mg combined gill and mantle (±10%) excised into 1 ml of Liftons lysis buffer (100 mM EDTA, 25 mM Tris-HCl, 1% SDS; pH 7.5) with 100 µg of Proteinase K. The level of tissue degradation in dead and moribund oysters varied considerably and, as such, the entirety of the remains of each animal were homogenised by a Stuart SHM1 handheld homogeniser with disposable blades. 50 µl of homogenate was added to 1 ml Liftons buffer (as above) with 100 µg of Proteinase K. Lysis buffer and tissue were incubated at 56°C for 1 hour with regular manual mixing (i.e. inverting the tube twice). Following digestion, 100 µl of lysate were added to 100 µl Qiagen ATL buffer

and processed with the Qiagen blood and tissue DNA extraction kit, following manufacturer's "tissue" protocol. DNA was quantified and quality checked by nanodrop.

2.4 Bacterial Quantification

Bacteria were quantified in each sample by qPCR according to methods adapted from Saulnier et al. (2017). Briefly, 2 µl of DNA were amplified in duplicate 20 µl multiplex reactions for detection of all bacteria from the *Vibrio* Splendidus clade (*V.* Splendidus polyphyletic group as defined by Sawabe et al. (2013) comprising eight species (*V. crassostreae, V. cyclitrophicus, V. gigantis, V. kanaloae, V. lentus, V. pomeroyi, V. splendidus, V. tasmaniensis*) (16S SpF2 5'-ATCATGGCTCAGATTGAACG-3', 16S SpR2 5'-CAATGGTTATCCCCCACATC-3', 16S probe 5'-FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1-3') and specific detection of *Vibrio aestuarianus* strains (DNAj F 5'-GTATGAAATTTTAACTGACCCACAA-3', DNAj R 5'-CAATTTCTTTCGAACAAC CAC-3', DNAj probe 5'-TexasRed-TGGTAGCGCAGACTTCGGCGAC-BHQ2-3') on a Roche lightcycler 480 Instrument II with 300 nM of each primer and 200 nM of probe. Samples were quantified against a standard curve of previously amplified and quantified pathogen DNA to give a measure equivalent to DNA quantity. Samples were analysed for the presence of OsHV-1 according to published protocol (Martenot et al., 2010). Data are reported as µg bacterial DNA per µl of DNA extraction.

2.5 Bacterial isolation

Live bacteria were isolated from frozen samples by homogenising a small portion of oyster gill (from live when sampled), or whole body (from dead when sampled) before serially diluting homogenate in sterile artificial seawater and plating on TCBS selective media (VWR Ltd., UK) or MA (Marine Agar; Marine 2216, Merck KGaA, Darmstadt, Germany)

(**Supplemental Figure 2**). Colonies were screened by qPCR to determine whether *V. Splendidus* or *V. aestuarianus* was present. However, we were only able to isolate *V. splendidus* using frozen oyster homogenates with TCBS or MA. In 2022, following removal of all travel restrictions, homogenates of tissue held on ice but not frozen were screened with TCBS and MA and we were able to isolate and identify *V. aestuarianus* colonies. These were slower growing, smaller and more translucent and lighter in colour compared with *V. Splendidus* colonies growing from the same homogenate.

2.6 DNA sequencing

Shotgun sequencing of total DNA from infected oysters were used to validate the presence of *Vibrio* spp. and identify any further potential infectious agents. 40 million 2x150 bp paired-end reads were generated using the Illumina NovaSeq platform (Genewiz, Leipzig, Germany). Illumina adapter removal and read filtration was performed using Trimmomatic (v 0.36) (Bolger et al., 2014) with the following parameters: TruSeq3-PE.fa:2:30:10:2:keepBothReads SLIDINGWINDOW:4:20 TRAILING:20 MINLEN:75. Reads were aligned in parallel to the reference assembly genomes for *C. gigas* (GCA_902806645.1) *V. aestuarianus* subsp. *francensis* (GCF_012395215.1) or *V. splendidus* (GCA_003050125.1) using the Burrows–Wheeler Aligner BWA-MEM (v 0.7.17) with default settings (Li, 2013). BAM files were generated, sorted and indexed with SAMtools (v 1.10) (Li et al., 2009). Coverage plots were generated using WGS coverage plotter (Pierre, 2015).

Long-read sequencing of the three isolated *V. splendidus* strains and the *V. aestuarianus* strain was performed at 100 – 300 x coverage using Oxford Nanopore Technologies MinION flow cells (R9.4.1) following the protocol for ligation sequencing (SQK-LSK109) and native barcoding (EXP-NBD104). Basecalling and barcode demultiplexing were performed using

Guppy (v 4.0.15). *De novo* assembly was performed using Flye (v 2.8.1) with two rounds of polishing using Racon (v 1.1) and Medaka (v 1.2). The final chromosome-level assemblies for each strain are available on NCBI GenBank under accession numbers GCA_022811945.1 (*V. splendidus* 1_C04a, best match type-strain: LMG 19031 (GCA_024347615.1) 96.41% Average Nucleotide Identity (ANI)), GCA_022812045.1 (*V. splendidus* 2_C04b, best match type-strain: LMG 19031 (GCA_024347615.1) 96.56% ANI), GCA_022812155.1 (*V. splendidus* 4_C08b, best match type-strain: LMG 19031 (GCA_024347615.1) 96.58% ANI) and BioProject ID PRJNA979146 (*V. aestuarianus* subsp*. francensis* LC_Sco). Phylogeny analysis was performed using the R Shiny app, Phy5 (Nakano et al., 2023) using the Euclidian distance matrix and complete agglomeration method parameters. Our three *V. splendidus* strains were compared against *V. splendidus* FF-6 (GCF_000272325.2), *V. splendidus* ZS_58 (GCF_003049845.1), *V. splendidus* FF_144 (GCF_003049965.1), *V. splendidus* ZS_173 (GCF_003050225.1), *V. splendidus* 1S_14 (GCF_003050485.1) and *V. splendidus* ZF_41 (GCF_003049865.1). Our *V. aestuarianus* assembly was compared against *V. aestuarianus* 02/041 (GCA_012395215.1), *V. aestuarianus* 07/115 (GCA_012689305.1), *V. aestuarianus* 01/308 (GCA_012689405.1), *V. aestuarianus* 10_092 7MT1 (GCA_028857075.1), *V. aestuarianus* 08_114_4T3 (GCA_028857095.1), *V. aestuarianus* SC_4077 (GCA_028857635.1), *V. aestuarianus* SC_3770 (GCA_028857675.1), *V. aestuarianus* SC_3670 (GCA_028857705.1) and *V. aestuarianus* MI_58_09 (GCA_028857745.1).

2.7 Blobtools analysis

To estimate microbial diversity of oyster mortality tissue sample, *de novo* contig assembly was performed using reads which did not map to the *C. gigas* assembly. Contigs were assembled using the SPAdes 3.12 –meta option (Prjibelski et al., 2020) and contigs >2k bases in

length were searched against the BLASTn database using the default parameters outlined by the Blobtools package (Laetsch and Blaxter, 2017).

2.8 Pathogen infection experiments

The pathogenicities of two *Vibrio splendidus* (*V.s._a* (C04a) and *V.s._b* (C04b)) strains and one *Vibrio aestuarianus* strain (*V.a.*) were tested in Pacific oysters obtained from a disease-free farm in the north-east of England. Bacteria were grown in liquid marine broth (Marine 2216, Merck KGaA, Darmstadt, Germany) to log phase before pelleting, cleaning with Artificial Sea Water (ASW; Tropic Marin Classic Sea Salt at a salinity of 35 ppt) and resuspending to the desired concentration (as below) in ASW just prior to challenge by injection directly into the adductor muscle with a 40-gauge needle. Oysters (shell height [40,50] mm, shell length [19,30] mm and shell depth [11,13] mm) were anaesthetised in ASW with 50 g/l $MgCl_2$ and without food (adapted from Suquet et al. (2009) for 2 hours prior to adductor injection with 50 ul of circa 10^6 or 10^7 CFU bacteria, or an ASW control. An initial small experiment to test the impact of temperature on single bacterial strain pathogenicity was done with animals injected with 10^6 CFU of each bacterial strain (*V.a.* and *V.s._a*, *V.a.* and *V.s._b*) and incubated individually in small 200 ml beakers (*n* = 4 per treatment), with 150 ml ASW, in the dark for 7 days at 15°C, 22°C or 25°C. Signs of morbidity or mortality were checked and recorded daily, and water changes done every two days. A second larger experiment with 5/6 oysters per treatment was performed in the same manner. Briefly, oysters (*n*=5 or *n*=6 per treatment) were held in individual tanks (5 x 5 x 10 cm), signs of morbidity or mortality were checked and recorded daily, and water changes done every two days. This second experiment was done at 14 °C and using both individual strains and combinations of bacteria (*V.a.* and *V.s._a*, Va and *V.s._b* or all three) at total injection quantities of 10^6 or 10^7 CFU/ml. Pathogen infection

experiment statistics were tested with pairwise Log-rank (Mantel-Cox) test in Graphpad prism (v 9.5.1).

3. Results

Levels of *V.* Splendidus, *V. aestuarianus* and OsHV-1 were quantified from all oyster samples and the two *Vibrio* clades quantified in environmental samples. *Vibrio* Splendidus were detected in live oysters, water and sediment in all samples with the exception of water in December. *Vibrio aestuarianus* was only detected from June onwards and was more frequently observed in the moribund / dead oyster samples (**Figure 1**). OsHV-1 was not detected in any of the analysed samples.

The appearance of *V. aestuarianus* followed increased temperatures, as did fluctuations in temperature coinciding with greater tidal ranges (**Supplemental Figure 1**).

To determine whether any organisms other than *Vibrio* were predominant in the moribund animals, we used shotgun sequencing on DNA extracted from an oyster tissue sample homogenate which showed *V. aestuarianus* infection by qPCR (**Supplemental Figure 3**). Reads from this sample aligned to the reference assembly genomes for *C. gigas, V. aestuarianus* subsp*. francensis* or *V. splendidus* (**Supplemental Figure 3**). Microbial diversity was assessed with contigs generated from all reads which did not map to the *C. gigas* assembly, the majority of which originated from *Vibrio* (**Supplemental Figure 4**) and mapped contiguously to the *V. aestuarianus* subsp*. francensis* assembly (**Supplemental Figure 3**).

We were able to isolate *Vibrio splendidus* colonies on both selective TCBS agar and unselective MA from frozen or chilled oyster tissue which had tested positive for this clade by qPCR. On TCBS, *V. splendidus* isolates formed either green colonies (C04a isolate) or orange colonies (C04b, C08b) (**Supplemental Figure 2**). *Vibrio aestuarianus* could only be recovered from chilled tissues on MA. Genome assemblies of these *V. splendidus and V. aestuarianus* isolates

were generated using Oxford Nanopore Technologies sequencing as described in the methods and reposited on NCBI (BioProject PRJNA764924). Phylogenetic analysis against other *V. splendidus* (Bruto et al., 2018) or *V. aestuarianus* species (Coyle et al., 2023; Mesnil et al., 2023), which had previously been associated with mortalities (**Figure 2**), shows that our isolate is of Lineage A, similar to Scottish isolates of *V. aestuarianus* used in previous studies (Coyle et al., 2023; Mesnil et al., 2023). Despite the green vs. orange phenotypes seen on the TCBS agar, our *V. splendidus* isolates appear more closely related to each other than the other *V. splendidus* isolates we analysed which have been associated with disease in *C. gigas*.

Finally, to test pathogenicity of these strains, we infected *C. gigas* individuals with our isolates of *V. aestuarianus* and both TCBS orange (V.s._b C04b) and TCBS green (V.s._b C04a) *V. splendidus* phenotypes. Pathogen infection experiments demonstrated that the *Vibrio aestuarianus* strain isolated was highly pathogenic at all tested temperatures and titres (**Figure 3** and **Figure 4**), causing up to 100% mortality in 2 days at 25 °C, 3 days at 22 °C, and in 6 days at 15 °C with an infection dose of 10^6 CFU. This was significantly different to V.s._b at all temperatures (p=0.01, p=0.0084 and p=0.0058 at 25 °C, 22 °C, and 15 °C respectively) and to V.s._a at the two lower temps (p=0.3173, p=0.0280 and p=0.0058 at 25 °C, 22 °C, and 15 °C, respectively). The individual *V. splendidus* strains however were only able to cause notable mortality at higher temperatures, or when present in combination with other strains. When the *V. splendidus* strains were presented alongside *V. aestuarianus* they were able to cause 80 – 100% mortality, but this did not differ significantly from *V. aestuarianus* alone at either of the tested titres (the greatest difference as observed between *V.s._a + V.a.* vs *V.a.* at 10^6 CFU was a difference in the time to reach 100% mortality, 6 days instead of 7 days (P = 0.6568). When *V. splendidus* strains were presented together at a higher titre without *V. aestuarianus* they were able to cause an intermediate level of mortality (50%), which was not

statistically different from the combinations including *V. aestuarianus* (V.s._a+V.s._b vs V.a at 10^7 CFUs, p=0.5439).

4. Discussion

Oyster growers globally are occasionally affected by large-scale mortality events which can be single or multi-factorial, including factors such as heat or salinity stress, or pathogens of viral, bacterial or parasitic nature. These events are often locally isolated but can on occasion affect larger regions. Here, we track an outbreak of *Vibrio aestuarianus* on a *Crassostrea gigas* farm in Scotland. The bacterial strain associated with mortalities at this farm proved highly pathogenic in laboratory experimental infections and phylogenetic analysis suggest it has previously been associated with mortality events occurring in France and Ireland (Coyle et al., 2023; Lupo et al., 2019; Mesnil et al., 2023).

Our qPCR analysis of live and dead oysters over three years revealed an increase in the prevalence of *V. aestuarianus* towards the end of the sampling period, and dead oysters, which were sampled opportunistically, also contained large amounts of *V. aestuarianus* DNA. Shotgun sequencing confirmed the predominance of *V. aestuarianus* in tissue from a moribund oyster sampled from the farm in August 2020. Only colonies of the *V.* Splendidus clade were recovered on TCBS agar using moribund tissue which contained high levels of *V. aestuarianus* DNA. This is in concurrence with previous reports that *V. aestuarianus* subsp. *francensis* does not grow well on TCBS, but does grow on MA, which has fewer selective properties (Garnier et al., 2008). Of note, we were only able to isolate *V. aestuarianus* from moribund animals delivered to the laboratory chilled on ice, never once from previously frozen tissue. Furthermore, when growing colonies on MA from cryopreserved stocks, *V.*

splendidus were visible after 24 hours, but *V. aestuarianus* colonies were only visible after 48 hours. Taken together, this suggests *V. aestuarianus* is more susceptible to cold/freezing and have more complex nutritional requirements than *V. splendidus*.

*Vibrio S*plendidus species have been implicated in *C. gigas* mortality events but has highly diverse virulence levels (Bruto et al., 2018), and as a species is well characterised as having many strains with different properties (Coyle et al. 2023). Experimental infections with the *V. splendidus* strains did not result in high mortality levels, in contrast to *V. aestuarianus,* indicating that *V. aestuarianus* was most likely responsible for the mortalities on the farm, perhaps exacerbated by the presence of opportunistic pathogens from the *V.* Splendidus clade.

Vibrio aestuarianus strains are known to display high phenotypic diversity, including a range of growth strategies and pathogenicity (Garcia et al., 2021; Garnier et al., 2008). Host, or site-specific pathogenicity has also been reported in *Vibrio* species, with some *Vibrio* strains having co-evolved with their *C. gigas* hosts over multiple generations of the same population in the same location (Wegner et al., 2019). Growers throughout the UK, however, source their farm stock from hatcheries, and move animals between farming locations to achieve the best growing conditions for each life stage. As these populations are not naturalised to the farm site, co-evolution is unlikely to take place between host and farm-specific microbes. Summer mortality has in the past been linked to reproductive stress, however, as is typical in a colder climate, the Pacific oysters farmed at this site do not reach sexual maturity (Samain, 2011). The appearance of mortality events due to pathogens such as *V. aestuarianus* most likely occurs through natural movement of the pathogen between sites, or concurrently with live stock movements. This is particularly important in naïve areas, such as Scotland, where they have the potential to cause significant mortality events without the potential for natural host

adaptation and, as things stand, without any legal requirement to put preventative measures in place. The ability to strictly classify *V. aestuarianus* subsp. *francensis* suggests it would have been possible, and potentially worthwhile, to track this strain and attempt to prevent spread into the UK. However, there have now been several independent isolations across the UK (Mesnil et al., 2023), suggesting that it is now endemic. As such, it is unlikely that measures put in place now would have significant impact on distribution.

While mortalities at this farm reached up to 50% in 2019, they did not exceed 15% in subsequent years from 2020 to 2022. Mortalities of 1-2% were recorded in the first half of 2020 but rose to 3-11% on average from September 2020 onwards. These mortalities were observed by a gaping/empty shell during grading/market harvest. Anecdotally, the rows of oysters which suffered the highest mortalities had undergone grading and/or were kept at a higher density than usual due to lack of sales during the height of the COVID pandemic in 2020. Samples of moribund animals from sporadic mortalities in 2020 – 2021 were used to further our research on these pathogenic bacteria and demonstrate persistence of the pathogenic strain in the years following initial mortalities, even though the same worrying levels of mortality observed in the initial outbreak were not repeated. Whilst *V. aestuarianus* has previously been incidentally reported in Scotland in studies exploring pathogen genetics and biology (Mesnil et al., 2023) this is the first detailed exploration of a mortality event in the region caused by this species.

Continued monitoring of this site throughout subsequent growing seasons should enable the identification of patterns of bacterial prevalence. For example, whilst samples were not collected, the farm manager reported very low (background) levels of mortality in 2023. Whilst this evidence is anecdotal, it is unusual for *V. aestuarianus* to be eradicated from a farm site and should the trend continue it perhaps deserves thorough investigation in coming

years. Data collected on other environmental variables such as rainfall and temperature could help indicate further stress from reduced salinity, low levels of dissolved oxygen and heat shock, which may coincide with mortality events. Additionally, continued isolation and sequencing of *V. aestuarianus* isolates will allow further comparison with isolates identified elsewhere throughout Europe (Garcia et al., 2021; Garnier et al., 2008) which can better inform potential transmission routes. Furthermore, histopathology performed on tissue from oysters with high levels of *V. aestuarianus* will inform whether *V. aestuarianus* infection is leading to the mortalities.

Whilst we have not been able to confirm these ideas by field challenges, this study has also inspired several suggestions of possible mitigation strategies. Mortalities occur around the same season each year, roughly when highest sea temperatures occur. As such, reducing unnecessary stress at this time, for example with an intermission in grading, may result in the reduction in mortality. As with most bacterial disease, temperature itself is likely one of the key drivers of disease. Our laboratory infection experiments demonstrate a positive relationship between higher temperatures and mortality, in concordance with previous field trials (Cowan et al., 2024), which perhaps suggests that moving oysters to cold regions prior to times of increased thermal stress could reduce pathogen impact. Notably, our laboratory studies also suggest that sensitivity to cold may be an important factor in the viability of *V. aestuarianus,* opening the possibility for exploring alternative treatment strategies. For example, Pacific oysters have a very strong cold tolerance and can survive acute exposure to sub-zero temperatures (Shatkin, 1997). As such, it is potentially realistic to perform short ice incubations during periods of oyster grading to reduce the viability of this pathogen, utilising the contrasting tolerances of *V. aestuarianus* and *C. gigas* to prevent disease. Finally, there was also the speculative observation that the initial mortality occurred whilst stocking higher

numbers of large animals (due to slow sales during COVID). Previous studies have also found that stocking density impacts outcome, with higher density stocking, and the resulting lower growth rate, seem to result in lower mortality (Cowan et al., 2024). The contribution of overall size and density in terms of numbers and biomass may be influencing mortality here and is something that can easily be tested.

In conclusion, we present the first published report of *V. aestuarianus* associated with a repeated mortality event at an oyster farm site in Scotland. Appearance of this species has been associated with mortalities across *C. gigas* growing sites elsewhere in Europe (Mesnil et al. 2023). Previous work suggests that *V. aestuarianus* is likely to persist in healthy oyster tissue rather than sediment during colder months (Parizadeh et al., 2018). As such, the appearance and persistence of this microbe is of interest to oyster growers across Scotland and in areas at risk of increasing sea temperatures due to climate change.

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Literature Cited

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15), 2114-2120. https://doi.org/10.1093/bioinformatics/btu170

Bruto, M., Labreuche, Y., James, A., Piel, D., Chenivesse, S., Petton, B., Polz, M.F., Le Roux, F., 2018. Ancestral gene acquisition as the key to virulence potential in environmental Vibrio populations. The ISME Journal 12(12), 2954-2966. https://doi.org/10.1038/s41396-018-0245-3

Cowan, M.W., Pearce, C.M., Green, T.J., Finston, T., Meyer, G.R., McAmmond, B., Van Hamme, J.D., Bottos, E.M., Marshall, R., Evans, W., Sutherland, T.F., de la Bastide, P.Y., 2024. Abundance of Vibrio aestuarianus, water temperature, and stocking density are associated with summer mortality of Pacific oysters in suspended culture. Aquaculture International. https://doi.org/10.1007/s10499-024-01415-5

Coyle, N.M., O'Toole, C., Thomas, J.C.L., Ryder, D., Feil, E.J., Geary, M., Bean, T.P., Joseph, A.W., Waine, A., Cheslett, D., Verner-Jeffreys, D.W., 2023. Vibrio aestuarianus clade A and clade B isolates are associated with Pacific oyster (Magallana gigas) disease outbreaks across Ireland. Microb Genom 9(8). https://doi.org/10.1099/mgen.0.001078

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., Haffner, P., Dégremont, L., Charrière, G.M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., Leroy, M.A., Pérignon, A., Régler, D., Morga, B., Alunno-Bruscia, M., Boudry, P., Le Roux, F., Destoumieux-Garzón, D., Gueguen, Y., Mitta, G., 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. Nature Communications 9(1), 4215. https://doi.org/10.1038/s41467-018-06659-3

Dégremont, L., Lamy, J.-B., Pépin, J.-F., Travers, M.-A., Renault, T., 2015. New Insight for the Genetic Evaluation of Resistance to Ostreid Herpesvirus Infection, a Worldwide Disease, in Crassostrea gigas. PLoS One 10(6), e0127917-e0127917. https://doi.org/10.1371/journal.pone.0127917

Dégremont, L., Morga, B., Maurouard, E., Travers, M.-A., 2021. Susceptibility variation to the main pathogens of Crassostrea gigas at the larval, spat and juvenile stages using unselected and selected oysters to OsHV-1 and/or V. aestuarianus. Journal of Invertebrate Pathology 183, 107601. https://doi.org/https://doi.org/10.1016/j.jip.2021.107601

Dégremont, L., Morga, B., Trancart, S., Pépin, J.-F., 2016. Resistance to OsHV-1 Infection in Crassostrea gigas Larvae. Frontiers in Marine Science 3(15). https://doi.org/10.3389/fmars.2016.00015
Divilov, K., Merz, N., Schoolfield, B., Green, T.J., Langdon, C., 2023. Genome-wide allele frequency studies in Pacific oyster families identify candidate genes for tolerance to ostreid herpesvirus 1 (OsHV-1). BMC Genomics 24(1), 631. https://doi.org/10.1186/s12864-023-09744-0
EFSA Panel on Animal Health & Welfare, 2010. Scientific Opinion on the increased mortality events in Pacific oysters, Crassostrea gigas. EFSA Journal 8(11), 1894.
FAO. 2024. The State of World Fisheries and Aquaculture 2024 – Blue Transformation in action. Rome.
Garcia, C., Mesnil, A., Tourbiez, D., Moussa, M., Dubreuil, C., Gonçalves de Sa, A., Chollet, B., Godfrin, Y., Dégremont, L., Serpin, D., Travers, M.-A., 2021. Vibrio aestuarianus subsp. cardii subsp. nov., pathogenic to the edible cockles Cerastoderma edule in France, and establishment of Vibrio aestuarianus subsp. aestuarianus subsp. nov. and Vibrio aestuarianus subsp. francensis subsp. nov. International Journal of Systematic and Evolutionary Microbiology 71(2). https://doi.org/https://doi.org/10.1099/ijsem.0.004654
Garnier, M., Labreuche, Y., Nicolas, J.-L., 2008. Molecular and phenotypic characterization of Vibrio aestuarianus subsp. francensis subsp. nov., a pathogen of the oyster Crassostrea gigas. Systematic and Applied Microbiology 31(5), 358-365. https://doi.org/https://doi.org/10.1016/j.syapm.2008.06.003
Gutierrez, A.P., Bean, T.P., Hooper, C., Stenton, C.A., Sanders, M.B., Paley, R.K., Rastas, P., Bryrom, M., Matika, O., Houston, R.D., 2018. A Genome-Wide Association Study for Host Resistance to Ostreid Herpesvirus in Pacific Oysters (Crassostrea gigas). G3 Genes|Genomes|Genetics 8(4), 1273-1280. https://doi.org/10.1534/g3.118.200113
Jennings, S., Stentiford, G.D., Leocadio, A.M., Jeffery, K.R., Metcalfe, J.D., Katsiadaki, I., Auchterlonie, N.A., Mangi, S.C., Pinnegar, J.K., Ellis, T., Peeler, E.J., Luisetti, T., Baker-Austin, C., Brown, M., Catchpole, T.L., Clyne, F.J., Dye, S.R., Edmonds, N.J., Hyder, K., Lee, J., Lees, D.N., Morgan, O.C., O'Brien, C.M., Oidtmann, B., Posen, P.E., Santos, A.R., Taylor, N.G.H., Turner, A.D., Townhill, B.L., Verner-Jeffreys, D.W., 2016. Aquatic food security: insights into challenges and solutions from an analysis of interactions between fisheries, aquaculture, food safety, human health, fish and human welfare, economy and environment. Fish and Fisheries 17(4), 893-938. https://doi.org/https://doi.org/10.1111/faf.12152
King, W.L., Siboni, N., Williams, N.L.R., Kahlke, T., Nguyen, K.V., Jenkins, C., Dove, M., O'Connor, W., Seymour, J.R., Labbate, M., 2019. Variability in the Composition of Pacific Oyster Microbiomes Across Oyster Families Exhibiting Different Levels of Susceptibility to OsHV-1 µvar Disease. Frontiers in Microbiology 10(473). https://doi.org/10.3389/fmicb.2019.00473
Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gélébart, F., Cordevant, C., Lange, M., Poulet, S.A., 2001. A Vibrio splendidus strain is associated with summer mortality of juvenile oysters Crassostrea gigas in the Bay of Morlaix (North Brittany, France). Dis Aquat Organ 46(2), 139-145. https://doi.org/10.3354/dao046139
Laetsch, D., Blaxter, M., 2017. BlobTools: Interrogation of genome assemblies [version 1; peer review: 2 approved with reservations]. F1000Research 6(1287). https://doi.org/10.12688/f1000research.12232.1
Lafont, M., Vergnes, A., Vidal-Dupiol, J., de Lorgeril, J., Gueguen, Y., Haffner, P., Petton, B., Chaparro, C., Barrachina, C., Destoumieux-Garzon, D., Mitta, G., Gourbal, B., Montagnani, C., 2020. A Sustained Immune Response Supports Long-Term Antiviral Immune Priming in the Pacific Oyster, Crassostrea gigas. mBio 11(2). https://doi.org/10.1128/mBio.02777-19
Laing, I., Walker, P., Areal, F., 2006. Return of the native – is European oyster (Ostrea edulis) stock restoration in the UK feasible? Aquatic Living Resources 19(3), 283-287. https://doi.org/10.1051/alr:2006029
Letchumanan, V., Chan, K.-G., Lee, L.-H., 2014. Vibrio parahaemolyticus: a review on the pathogenesis, prevalence, and advance molecular identification techniques. Frontiers in microbiology 5, 705-705. https://doi.org/10.3389/fmicb.2014.00705

Li, H., 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint arXiv:1303.3997.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., Subgroup, G.P.D.P., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352
Lupo, C., Travers, M.-A., Tourbiez, D., Barthélémy, C.F., Beaunée, G., Ezanno, P., 2019. Modeling the Transmission of Vibrio aestuarianus in Pacific Oysters Using Experimental Infection Data. Frontiers in Veterinary Science 6(142). https://doi.org/10.3389/fvets.2019.00142
Martenot, C., Oden, E., Travaillé, E., Malas, J.P., Houssin, M., 2010. Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster Crassostrea gigas. J Virol Methods 170(1-2), 86-89. https://doi.org/10.1016/j.jviromet.2010.09.003
Mesnil, A., Jacquot, M., Garcia, C., Tourbiez, D., Canier, L., Bidois, A., Dégremont, L., Cheslett, D., Geary, M., Vetri, A., Roque, A., Furones, D., Garden, A., Orozova, P., Arzul, I., Sicard, M., Charrière, G.M., Destoumieux-Garzón, D., Travers, M.-A., 2023. Emergence and clonal expansion of Vibrio aestuarianus lineages pathogenic for oysters in Europe. Molecular Ecology 32(11), 2869-2883. https://doi.org/https://doi.org/10.1111/mec.16910
Nakano, Y., Domon, Y., Yamagishi, K., 2023. Phylogenetic trees of closely related bacterial species and subspecies based on frequencies of short nucleotide sequences. PLoS One 18(4), e0268847. https://doi.org/10.1371/journal.pone.0268847
Parizadeh, L., Tourbiez, D., Garcia, C., Haffner, P., Dégremont, L., Le Roux, F., Travers, M.-A., 2018. Ecologically realistic model of infection for exploring the host damage caused by Vibrio aestuarianus. Environmental Microbiology 20(12), 4343-4355. https://doi.org/https://doi.org/10.1111/1462-2920.14350
Pierre, L., 2015. JVarkit: java-based utilities for Bioinformatics.
Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., Korobeynikov, A., 2020. Using SPAdes De Novo Assembler. Current Protocols in Bioinformatics 70(1), e102. https://doi.org/https://doi.org/10.1002/cpbi.102
Regan, T., Bean, T.P., Ellis, T., Davie, A., Carboni, S., Migaud, H., Houston, R.D., 2021. Genetic improvement technologies to support the sustainable growth of UK aquaculture. Reviews in Aquaculture 13(4), 1958-1985. https://doi.org/https://doi.org/10.1111/raq.12553
Renault, T., Bouquet, A.L., Maurice, J.-T., Lupo, C., Blachier, P., 2014. Ostreid herpesvirus 1 infection among Pacific oyster (Crassostrea gigas) Spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. Appl Environ Microbiol 80(17), 5419-5426. https://doi.org/10.1128/AEM.00484-14
Saulnier, D., De Decker, S., Tourbiez, D., Travers, M.A., 2017. Development of a duplex Taqman real-time PCR assay for rapid identification of Vibrio splendidus-related and V. aestuarianus strains from bacterial cultures. Journal of Microbiological Methods 140, 67-69. https://doi.org/https://doi.org/10.1016/j.mimet.2017.07.002
Samain, J.F., 2011. Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster Crassostrea gigas to summer mortality. Aquatic Living Resources, 24(3), 227-236. https://doi.org/10.1051/alr/2011144

Sawabe, T., Ogura, Y., Matsumura, Y., Feng, G., Amin, A.R., Mino, S., Nakagawa, S., Sawabe, T., Kumar, R., Fukui, Y., Satomi, M., Matsushima, R., Thompson, F.L., Gomez-Gil, B., Christen, R., Maruyama, F., Kurokawa, K., Hayashi, T., 2013. Updating the Vibrio clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of Vibrio tritonius sp. nov. Front Microbiol 4, 414. https://doi.org/10.3389/fmicb.2013.00414

Shatkin, G., 1997. Considerations regarding the possible introduction of the Pacific oyster (Crassostrea gigas) to the Gulf of Maine: a review of global experience. J. Shellfish Res. 16, 463-478.
Sihvonen, L.H., 2015. Scientific opinion on oyster mortality. EFSA Journal 13(6), 4122.

Suquet, M., de Kermoysan, G., Araya, R.G., Queau, I., Lebrun, L., Le Souchu, P., Mingant, C., 2009. Anesthesia in Pacific oyster, Crassostrea gigas. Aquat. Living Resour. 22(1), 29-34.
Wegner, K.M., Piel, D., Bruto, M., John, U., Mao, Z., Alunno-Bruscia, M., Petton, B., Le Roux, F., 2019. Molecular Targets for Coevolutionary Interactions Between Pacific Oyster Larvae and Their Sympatric Vibrios. Frontiers in Microbiology 10(2067). https://doi.org/10.3389/fmicb.2019.02067

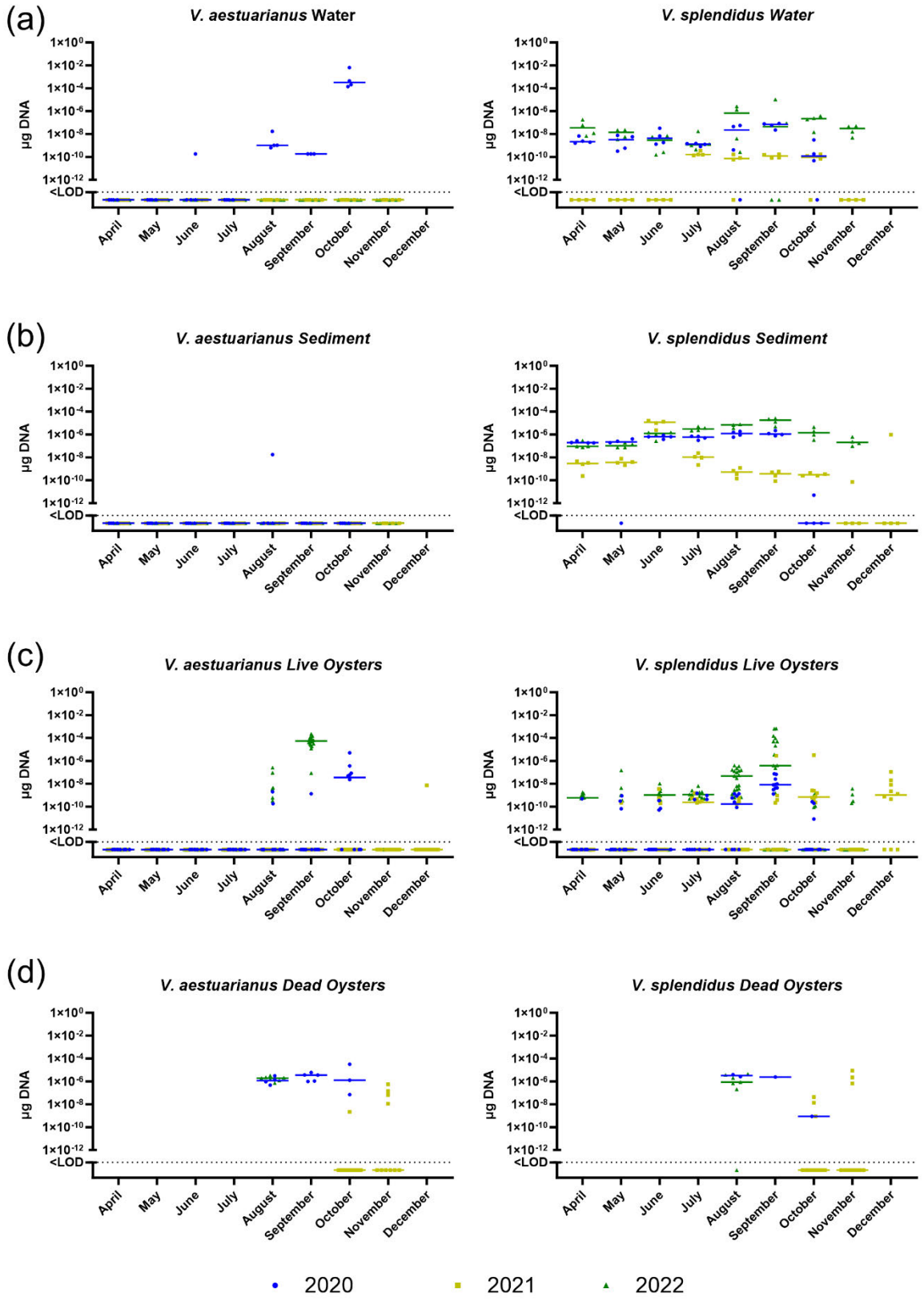


Figure 1 PCR quantification of DNA from *Vibrio aestuarianus* or *Vibrio splendidus* as indicated from individual samples of (a) Water, (b) Sediment, (c) Live oysters or (d) Dead oysters isolated once per month from the farm site. Quantification denotes bacterial µg per µl of DNA extract.

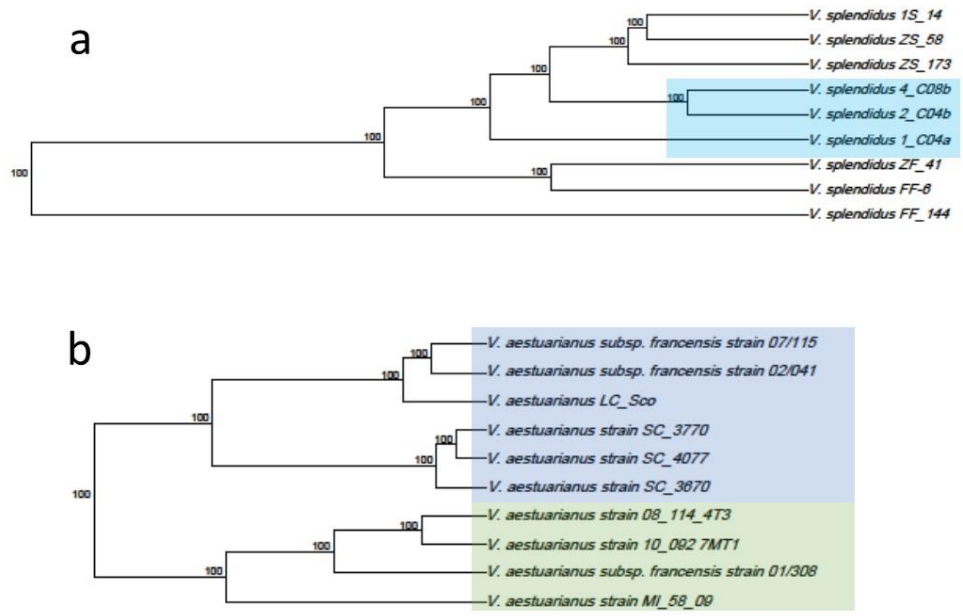


Figure 2 *Vibrio splendidus* and *Vibrio aestuarianus* subsp*. francensis* relatedness. Genome assemblies of (a) *V. splendidus* or (b) *V. aestuarianus* from NCBI RefSeq were compared using a Euclidian distance matrix, complete agglomeration method (Phy5R). *Vibrio splendidus* isolates from this study are shown in (a) cyan Lineage A (blue) and (b) Lineage B (green) are highlighted for *V. aestuarianus*.

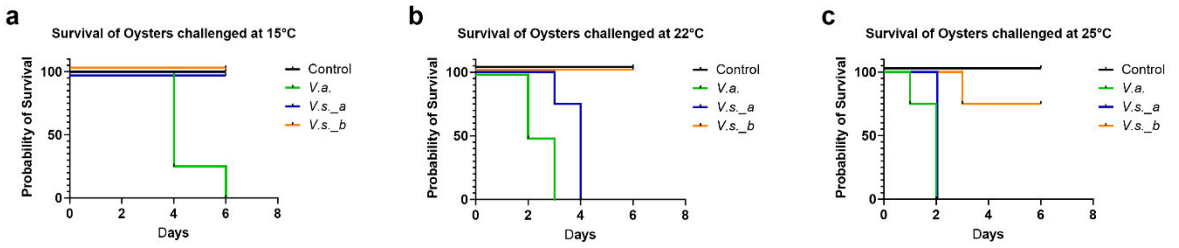


Figure 3 Survival of oysters challenged with isolated *Vibrio* species. Oysters were subjected to a 7 day infection challenge with either *V. aestuarianus* (*V.a.*), *V. splendidus* C04a (*V.s._a*) or *V. splendidus* C04b (*V.s._b*) via adductor muscular injection at a total concentration of 10^6 CFU and held in artificial seawater at the temperatures shown: (a) 15°C, (b) 22°C or (c) 25°C.

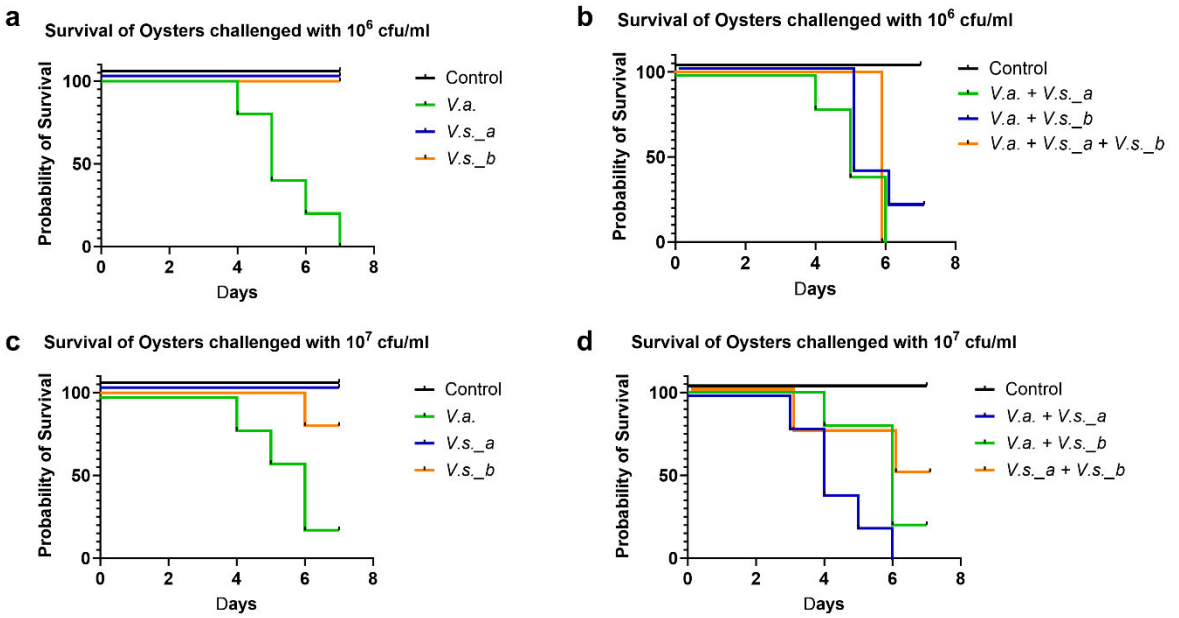
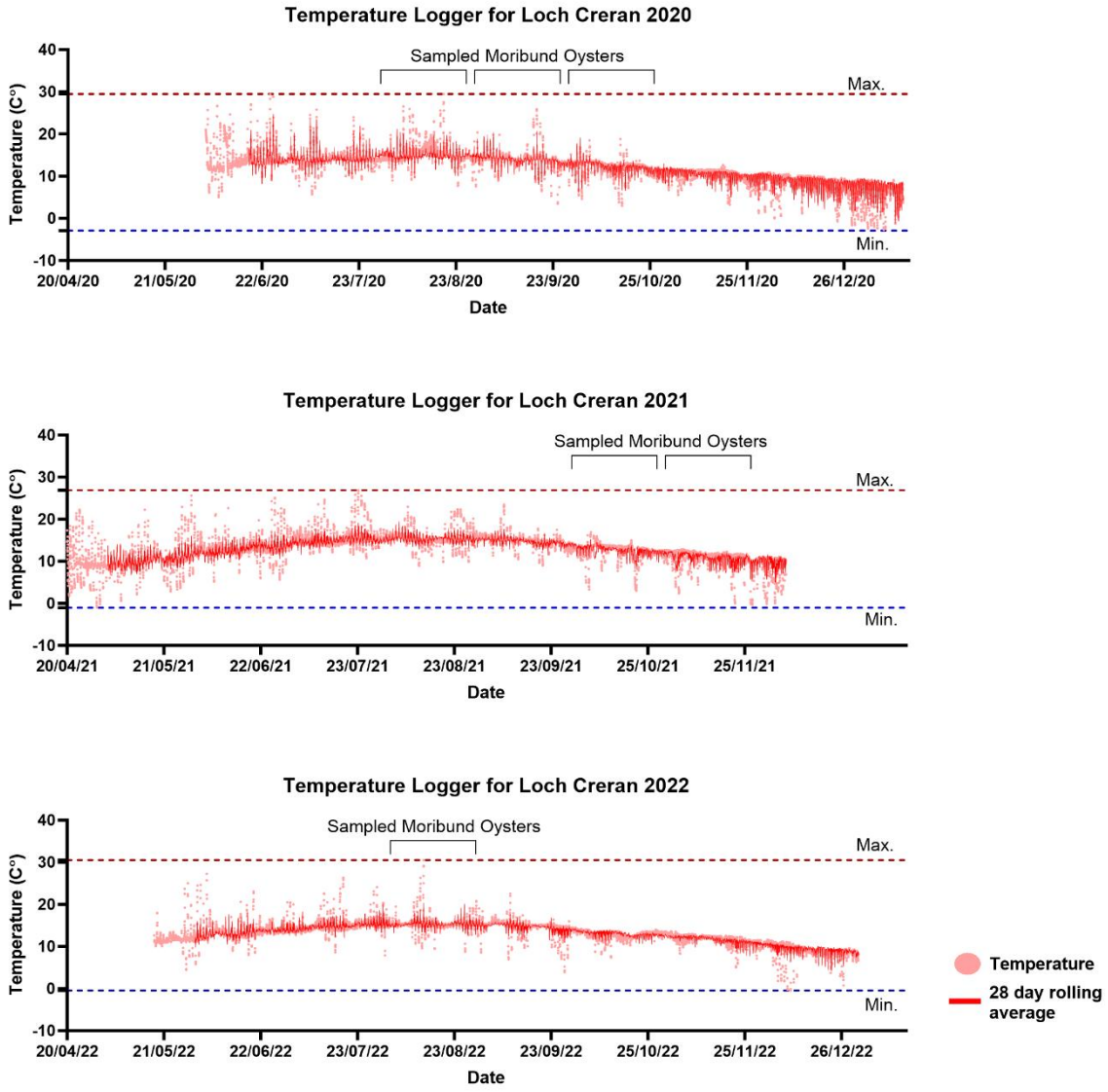


Figure 4 Survival of oysters challenged with isolated *Vibrio* species. Oysters were subjected to a 7 day infection challenge. Animals were held in artificial seawater at 14°C with either *V. aestuarianus* (*V.a.*), *V. splendidus* C04a (*V.s._a*) or *V. splendidus* C04b (*V.s._b*) or combinations of these strains added via adductor muscular injection at a total concentration of either (a, b) 10^6 CFU or (c, d) 10^7 CFU as shown.

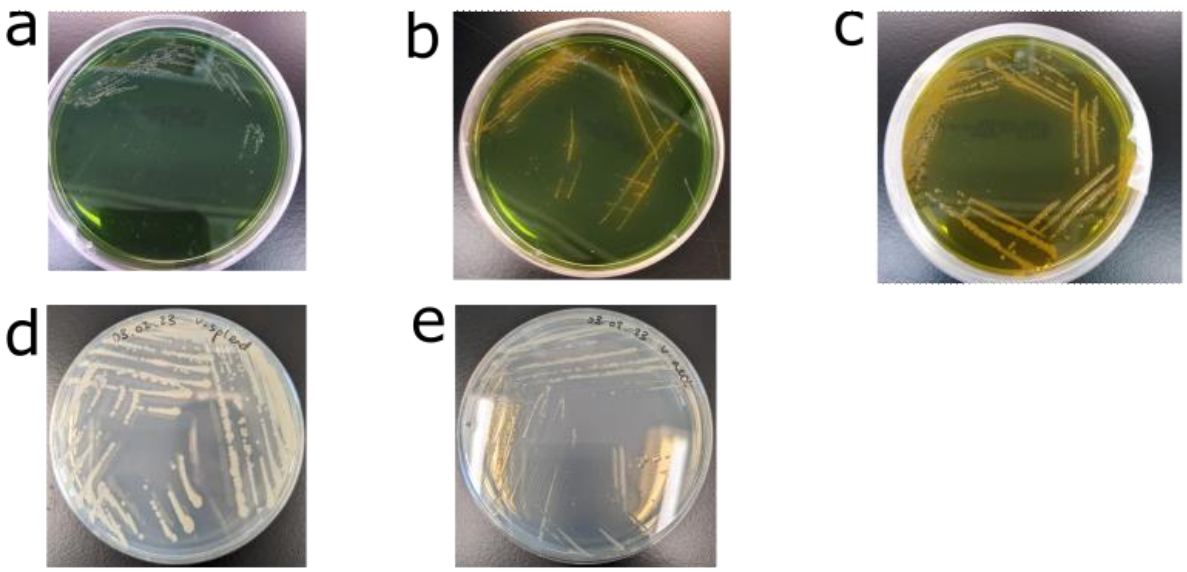
Table S1 Samples used in the study including date and source of sampling: oyster (live); oyster (dead); water; sediment.

Sample	Source	Date
W2-1	Water	2020-04-26
W2-2	Water	2020-04-26
W2-3	Water	2020-04-26
W2-4	Water	2020-04-26
W5-1	Water	2020-05-26
W5-2	Water	2020-05-26
W5-3	Water	2020-05-26
W5-4	Water	2020-05-26
W8-1	Water	2020-06-25
W8-2	Water	2020-06-25
W8-3	Water	2020-06-25
W8-4	Water	2020-06-25
W11-1	Water	2020-07-24
W11-2	Water	2020-07-24
W11-3	Water	2020-07-24
W11-4	Water	2020-07-24
W16-1	Water	2020-08-24
W16-2	Water	2020-08-24
W16-3	Water	2020-08-24
W16-4	Water	2020-08-24
W21-1	Water	2021-09-20
W21-2	Water	2021-09-20
W21-3	Water	2021-09-20
W21-4	Water	2021-09-20
W28-1	Water	2020-10-20
W28-2	Water	2020-10-20
W28-3	Water	2020-10-20
W28-4	Water	2020-10-20
S1-1	Sediment	2020-04-26
S1-2	Sediment	2020-04-27
S1-3	Sediment	2020-04-28
S1-4	Sediment	2020-04-29
S4-1	Sediment	2020-04-30
S4-2	Sediment	2020-05-01
S4-3	Sediment	2020-05-02
S4-4	Sediment	2020-05-03
S7-1	Sediment	2020-05-04
S7-2	Sediment	2020-05-05
S7-3	Sediment	2020-05-06
S7-4	Sediment	2020-05-07
S10-1	Sediment	2020-05-08
S10-2	Sediment	2020-05-09
S10-3	Sediment	2020-05-10
S10-4	Sediment	2020-05-11
S15-1	Sediment	2020-05-12
S15-2	Sediment	2020-05-13
S15-3	Sediment	2020-05-14
S15-4	Sediment	2020-05-15
S20-1	Sediment	2020-05-16
S20-2	Sediment	2020-05-17
S20-3	Sediment	2020-05-18
S20-4	Sediment	2020-05-19
S29-1	Sediment	2020-05-20
S29-2	Sediment	2020-05-21
S29-3	Sediment	2020-05-22
S29-4	Sediment	2020-05-23

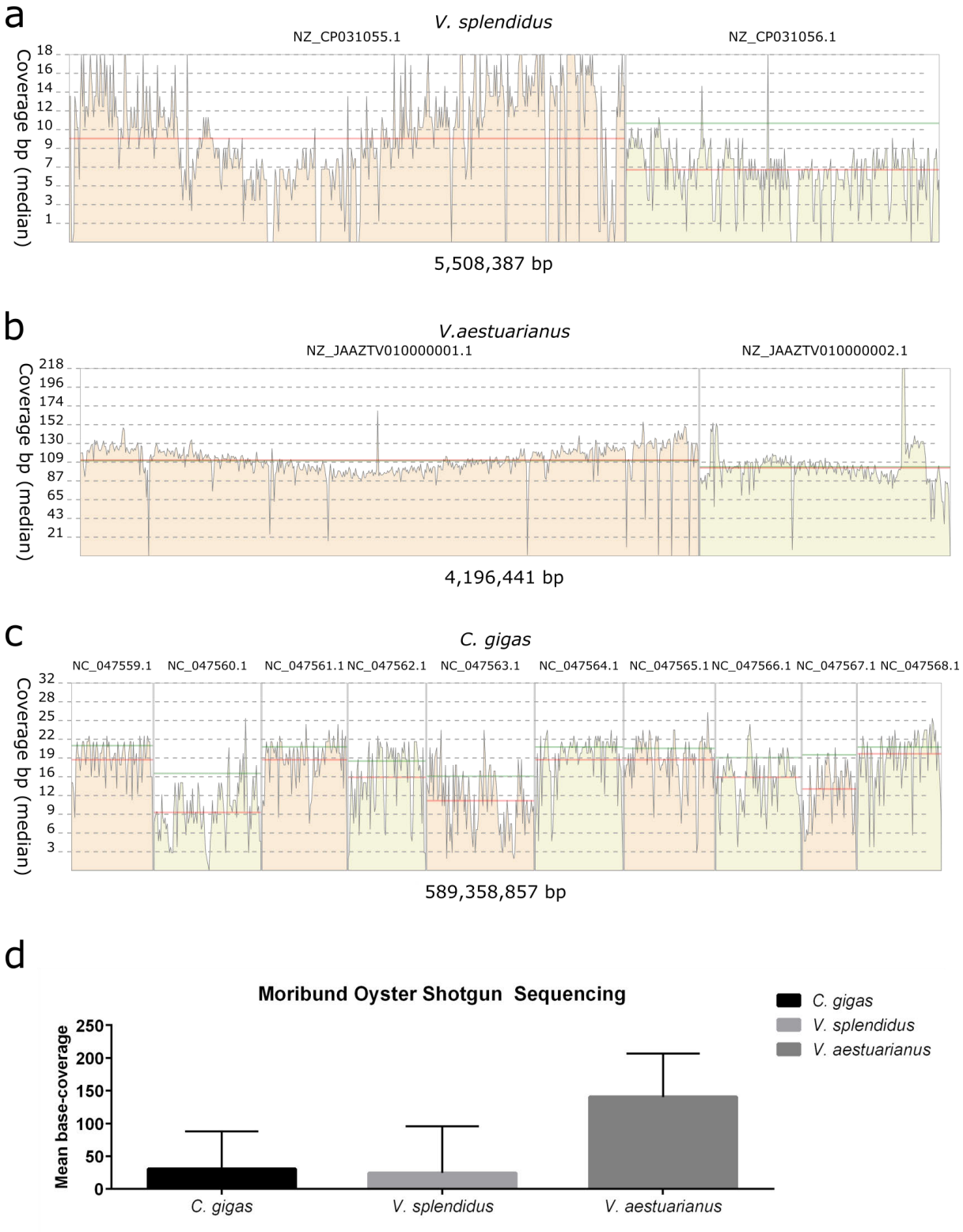
Sample	Source	Date
O3-1	Oyster/Live	2020-04-26
O3-2	Oyster/Live	2020-04-27
O3-3	Oyster/Live	2020-04-28
O3-4	Oyster/Live	2020-04-29
O3-5	Oyster/Live	2020-04-30
O3-6	Oyster/Live	2020-05-01
O6-1	Oyster/Live	2020-05-02
O6-2	Oyster/Live	2020-05-03
O6-3	Oyster/Live	2020-05-04
O6-4	Oyster/Live	2020-05-05
O6-5	Oyster/Live	2020-05-06
O6-6	Oyster/Live	2020-05-07
O9-1	Oyster/Live	2020-05-08
O9-2	Oyster/Live	2020-05-09
O9-3	Oyster/Live	2020-05-10
O9-4	Oyster/Live	2020-05-11
O9-5	Oyster/Live	2020-05-12
O9-6	Oyster/Live	2020-05-13
O12-1	Oyster/Live	2020-05-14
O12-2	Oyster/Live	2020-05-15
O12-3	Oyster/Live	2020-05-16
O12-4	Oyster/Live	2020-05-17
O12-5	Oyster/Live	2020-05-18
O12-6	Oyster/Live	2020-05-19
O13-1	Oyster/Live	2020-05-20
O13-1	Oyster/Live	2020-05-21
O13-1	Oyster/Live	2020-05-22
O13-2	Oyster/Live	2020-05-23
O22-4	Oyster/Live	2020-06-10
O22-5	Oyster/Live	2020-06-11
O22-6	Oyster/Live	2020-06-12
O22-7	Oyster/Live	2020-06-13
O22-8	Oyster/Live	2020-06-14
O22-9	Oyster/Live	2020-06-15
O30-1	Oyster/Live	2020-06-16
O30-2	Oyster/Live	2020-06-17
O30-3	Oyster/Live	2020-06-18
O30-4	Oyster/Live	2020-06-19
O30-5	Oyster/Live	2020-06-20
O30-6	Oyster/Live	2020-06-21
OM18-1	Oyster/Mort.	24/08/2020
OM18-2	Oyster/Mort.	25/08/2020
OM19-1	Oyster/Mort.	26/08/2020
OM19-2	Oyster/Mort.	27/08/2020
OM23	Oyster/Mort.	28/08/2020
OM24	Oyster/Mort.	29/08/2020
OM25	Oyster/Mort.	30/08/2020
OM26	Oyster/Mort.	31/08/2020
OM27	Oyster/Mort.	01/09/2020
OM31	Oyster/Mort.	02/09/2020
OM32	Oyster/Mort.	03/09/2020
OM33	Oyster/Mort.	04/09/2020



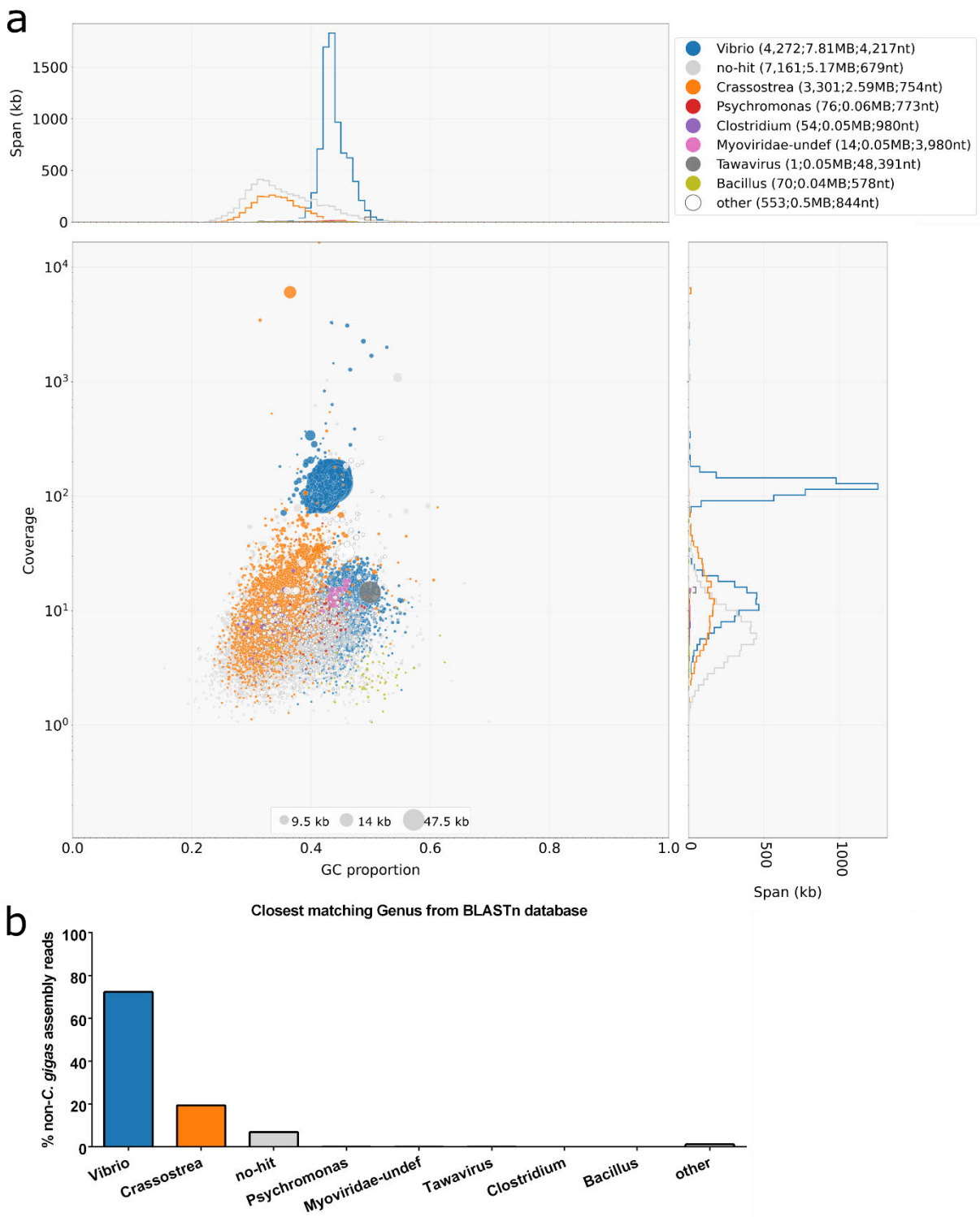
Supplemental Figure 1 Temperature recorded on farm site as discrete 30-minute interval observations (pink) or as a 28-day rolling average (red). Max and min lines denote highest and lowest records of the year. Sampled Moribund Oysters denotes the period during which mortalities were observed and sampled.



Supplemental Figure 2 Isolates of (a-c) *Vibrio splendidus* from homogenized oyster tissue grown on TCBS agar and (d) marine agar and (e) *Vibrio aestuarianus* grown on marine agar.



Supplemental Figure 3 Alignment of DNA isolated from a moribund oyster. Shotgun sequencing reads were aligned to the reference assemblies for (a) *Vibrio splendidus* (GCA_003050125.1), (b) *V. aestuarianus* (GCF_012395215.1) and (c) *Crassostrea gigas* (GCA_902806645.1). Chromosomes are differentiated by alternating orange and green colours. (d) The mean base coverage for each assembly is also shown (error bars represent standard deviation).



Supplemental Figure 4 Blobtools output for genus level matches from BLASTn of *de novo* assembled contigs using shotgun reads not mapping to the *Crassostrea gigas* assembly. (a) Contigs, coloured according to genus, are represented as "blobs". The size of each blob is relative to contig length. Cumulative contig span is shown for GC content and coverage on the x- and y-axes respectively. Shown in (b) is percentage of reads assigned to each genus of the reads which did not map to the *C. gigas* assembly.